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VERTICILLIUM WILT OF AVOCADO[1]

GEORGE A. ZENTMYER[2]

(Accepted for publication March 24, 1949)

In the past 15 to 20 years there have been occasional reports of sudden wilting and collapse of isolated avocado trees in otherwise healthy groves in California. This trouble, which was thought to be connected with a lack of oxygen in the soil, brought about by a sudden saturation of the soil with

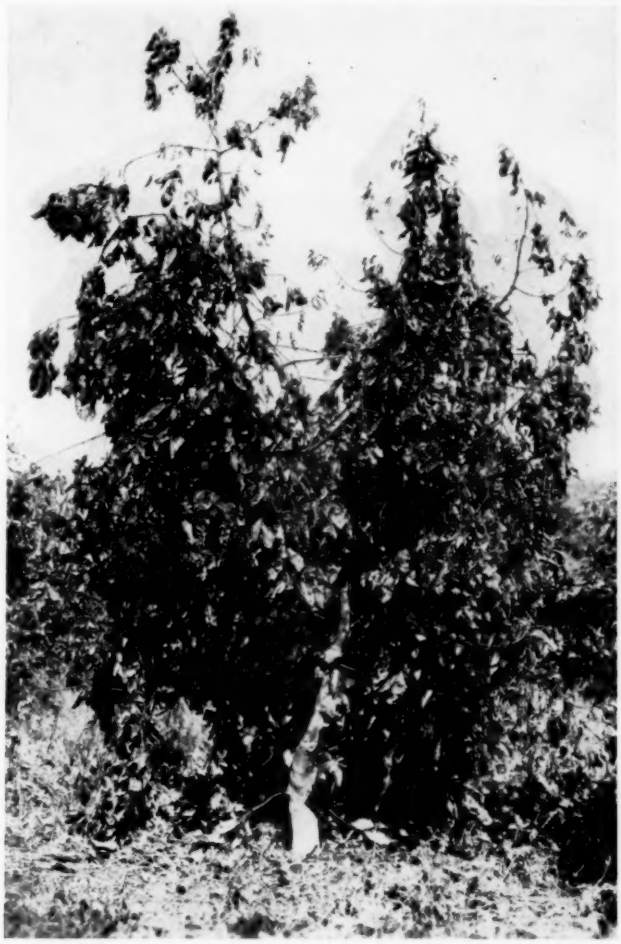

FIG. 1. Fuerte avocado tree affected with Verticillium wilt. The entire top wilted suddenly, and the leaves died and turned brown. A few new shoots are developing from the trunk.

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water, has been variously termed collapse, asphyxiation, and apoplexy (1, 3). Investigations during the winter of 1947–1948, reported in the present paper, showed that this disease is caused by the fungus *Verticillium albo-atrum* Reink. and Berth.

SYMPTOMS

The symptoms of Verticillium wilt on avocado are similar to those on other woody hosts including maple, elm, apricot, and peach. There is usually a sudden wilting of the leaves on one branch, on one side of a tree, or on an entire tree, followed quickly by death of the leaves (Fig. 1). The leaves turn brown and remain on the tree for several months.

At the time of the initial wilting and "collapse," dark-brown streaks are readily seen in the most recently formed xylem tissue when the bark is peeled from affected branches or from the trunk. Streaks may be abundant, covering most of the circumference of the branch (Fig. 2), or may be few in number.

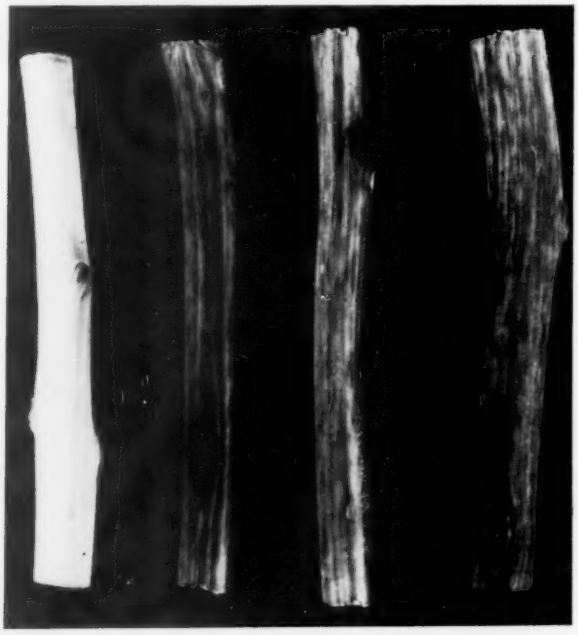

FIG. 2. Healthy avocado branch (left) and three diseased branches (right), from which bark has been peeled, showing vascular discoloration (brown) caused by *Verticillium albo-atrum*.

Dieback occurs in varying degrees, and occasionally an entire tree is killed. More commonly, there is dieback of one-third to two-thirds of the top; then, a month or more after the initial wilting and death of leaves, new shoots appear from adventitious buds along the larger branches and the trunk, and vigorous new growth rapidly regenerates a new top. This renewal of growth coincides with, or closely follows, formation of new xylem tissue replacing that invaded by the fungus.

After the initial appearance of the disease, trees often completely recover and do not wilt again. Trees may wilt several years in succession, however. The disease has been observed in December, January, June, and July.

THE CAUSAL FUNGUS

Verticillium albo-atrum was first isolated from avocado trees at Vista, San Diego County, California, in December, 1947. This isolate and subsequent isolates from a number of other diseased trees were typical of *V. albo-atrum* as described by Reinke and Berthold and as clarified by Rudolph (4).[3]

Wilhelm (5) states that the conidial constituent of *V. albo-atrum* forms abundant black microsclerotia at temperatures of 10° to 22° C., while at 25° to 31° C. the colonies are creamy-white, with few microsclerotia. He further states that the stable white mycelial variants are unaffected by temperature. In the present experiments, the isolates from avocado showed the same responses to temperature as those noted by Wilhelm.

VARIETIES AND LOCATION OF AVOCADO TREES AFFECTED

Under natural conditions Verticillium wilt has been found on Guatemalan seedling trees (*Persea americana* Miller), on Guatemalan varieties (Anaheim and Queen) budded on Guatemalan rootstocks, and on the hybrid Fuerte variety budded on Guatemalan and Mexican (*P. americana* var. *drymifolia* (Schlechtendal and Chamisso) Blake) rootstocks. The rootstock types were determined by the method developed by Halma (2).

Various Mexican varieties comprise the majority of the rootstock sources for commercial avocado trees in California, although some Guatemalan seedlings have been used. The Fuerte is the principal variety under commercial production in California.

The disease has been found in all of the commercial avocado districts in California, including sections of San Diego, Orange, Los Angeles, Ventura, and Santa Barbara counties. Trees naturally infected have ranged from 2½ to 20 years of age. Since the disease usually occurs on only one or two isolated trees in a grove, it is not at present of economic importance.

INOCULATION

Avocado seedlings were inoculated in the greenhouse by two main methods: either by dipping roots in a suspension of spores and mycelium, or by injecting spores into the vascular system of the stem.

For inoculation by the first method, two 14-day-old Petri-dish cultures of *V. albo-atrum*, isolated from avocado trees and growing on potato-dextrose agar, were homogenized with water in a Waring Blendor. The roots of ten 10-month-old seedlings, five each of Mexican (Topa-Topa) and Guatemalan

[3] The isolate from avocado was submitted for examination to Drs. B. A. Rudolph and S. Wilhelm of the University of California at San Jose and Berkeley, and was identified as *V. albo-atrum*.

(Anaheim), were dipped in the spore-mycelium suspension and planted in cans of sterilized greenhouse soil (Fig. 3), which were placed in temperature tanks, one each at 15°, 20°, 25°, 30°, and 35° C., in August, 1948. Tomato plants (Improved Pearson) were also inoculated by dipping the roots in the suspension and were planted in cans placed in the temperature tanks. Soil temperatures were maintained at 15°, 20°, 25°, 30°, and 35° C.

FIG. 3. Guatemalan (left) and Mexican (right) avocado seedlings inoculated by dipping roots in spore and mycelium suspension of *Verticillium albo-atrum*. Photographed 4 weeks after inoculation.

Twelve days after inoculation the first symptoms of Verticillium wilt appeared in three Guatemalan seedlings, one each at 20°, 25°, and 30° C., from all of which the pathogen was recovered. These seedlings wilted suddenly, and the leaves died within another week. Vascular discoloration was present in leaf petioles, and the fungus was recovered from the petioles. Disease appearance was delayed in this variety when grown at 15° C., and no symptoms appeared at 35° C.

The Mexican-type (Topa-Topa) seedlings were not so severely affected by the inoculation as the Guatemalan. Moderate wilting of the leaves oc-

curred in the tanks held at 20°, 25°, and 30° C., 3 to 4 weeks after inoculation. Light vascular discoloration was present in the seedlings grown at these three soil temperatures, and also in those grown at 15° and 35° C. Plants grown at these two temperatures had no other symptoms of the disease, except a slight wilt of leaves at 35° C.

After the death and abscission of some of the leaves, new shoots appeared on both types of seedlings grown at 20°, 25°, and 30° C., as under conditions of natural infection. No symptoms developed on either Mexican or Guatemalan control seedlings.

Inasmuch as a rather resistant variety of tomato was used, symptoms were not obvious on these plants; there was some one-sided yellowing of leaves, however, and *V. albo-atrum* was reisolated from the faintly discolored portions of the petioles and stem.

Two Anaheim and two Topa-Topa seedlings were inoculated by the second method, which has been used successfully in studies on the Dutch elm disease and other vascular diseases (6). The stem of the avocado seedling was placed in a horizontal position, a drop of concentrated spore suspension was pipetted onto the stem, and the drop was cut through with a scalpel, the cut penetrating into the xylem. The spore suspension is readily taken up by the vessels in this method. Ten days later several leaves in the top of both Anaheim seedlings and one Topa-Topa seedling showed a one-sided yellowing and a drying-out of tissue. Vascular stain was heavy, and the fungus was readily reisolated from leaf petioles in the top of the 18-in. seedlings.

PREVENTIVE MEASURES

The causal fungus in many cases does not survive in the invaded wood of the avocado for any appreciable length of time. It is readily isolated from branches at the time of or soon after the initial appearance of disease symptoms, but a few months later it is often impossible to isolate the fungus, even from heavily stained wood. Similar difficulty was noted in reisolation from the artificially inoculated seedlings. This may be correlated with the fact that many trees apparently recover completely from the disease. Recurrence of the disease on some trees for several years in succession may indicate a particularly susceptible rootstock. Such trees should be removed from the grove, and if the area is to be replanted to avocado, the soil should be fumigated with chloropicrin before replanting.

Obvious measures to reduce the possibility of disease occurrence include not planting avocado trees on land that has been recently cropped to tomatoes or other plants highly susceptible to *Verticillium albo-atrum*. Several groves in which the disease has appeared were planted to tomatoes the year before the avocado trees were set out. In the case of nursery plantings, the use of land that has been in some other crop susceptible to *V. albo-atrum* should be particularly avoided. Interplanting young groves with susceptible crops should also be avoided.

SUMMARY

The causal agent of a disease of avocado trees in California involving sudden wilting and collapse of the leaves has been identified as *Verticillium albo-atrum* Reink. and Berth. Trees usually are not killed entirely and often make excellent recovery.

The disease is present in all of the commercially producing avocado districts in southern California. Trees on both Mexican and Guatemalan rootstocks are affected. The disease had been found on both seedlings and budded trees.

Experimental seedlings inoculated by dipping the roots in a suspension of spores and mycelium showed disease symptoms in 12 days at soil temperatures of 20°, 25°, and 30° C. The Guatemalan seedlings were more severely affected by the inoculation than the Mexican.

Certain preventive measures are suggested.

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EFFECTS OF BACTERIUM TUMEFACIENS ON ALLIUM CEPA[1]

SOPHIE JAKOWSKA[2]

(Accepted for publication March 1, 1949)

INTRODUCTION

Bacterium tumefaciens has been used experimentally not only to infect plants which normally succumb to it in their natural habitat but also to test the susceptibility of some plants which are ordinarily not infected by *B. tumefaciens*. The work of Smith and Quirk (36) on *Begonia*, which was immune from *B. tumefaciens*, gave the incentive to the study of other cases of unusual infection. *Allium cepa* was listed by these authors (36) as a plant unlikely to become infected because of the unfavorable hydrogen-ion concentration of the sap. Since no reports of *B. tumefaciens* infection of *A. cepa* had been made, it seemed desirable to test the susceptibility of this plant to *B. tumefaciens* with the hope of producing some form of crown gall on either the young seedling or on the adult bulb. There was also the hope that it might be possible to study such overgrowths from the viewpoints of general morphology and cytology.

The cytological effects of *Bacterium tumefaciens* infection were, if they existed, to be of considerable interest in view of the fact that cytological studies of the crown gall disease are restricted to the rather limited data from Smith, Brown, and McCulloch (35), Robinson and Walkden (33), Levine (21, 23, 24), Riker (30), Riker and Berge (31), and Winge (39). Winge's paper is the most extensive. His cytological information is not incidental but constitutes the subject of the investigation. Winge concluded that *B. tumefaciens* acts on living cells and that polyploid cells are found in galls caused by *B. tumefaciens*. However, reports of the actual presence of *B. tumefaciens* in living cells have been few and the evidence on the subject still is contradictory in view of the micrurgical studies of Hildebrand (11), who concluded that a single *B. tumefaciens* is unable to survive and reproduce after introduction into a living cell. More and more evidence is accumulating in favor of the view that auxin-like substances are produced by *B. tumefaciens*, and this theory would of course allow for the action of *B. tumefaciens* apart from its actual entrance into the living cell. It is still undetermined, however, whether the chromosomal constitution of cells involved in crown gall formation is an essential feature of infection by *B. tumefaciens*. In material susceptible to *B. tume-*

[1] Part of a dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Biology at Fordham University, New York City, N. Y., June, 1947. The author expresses her thanks to Dr. E. R. Witkus, under whose direction this work was completed.

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faciens, the study of abnormal mitosis with consequent changes in number of chromosomes failed to reach any degree of certainty, because size of chromosomes was the main limiting factor. For this reason, the attempt was made to infect with *B. tumefaciens* a plant whose normal cytology is well known, having large chromosomes which have been observed under a wide variety of experimental conditions.

The cytological approach to the problem of crown gall has been abandoned in most respects during recent years, but among the authors who have treated in detail cytological phenomena in plant tumors are Winge (39) in his work on the sugar-beet crown gall, Whitaker (37), who studied spontaneous tumors of *Nicotiana* hybrids, and Wipf (40), who investigated root nodules of Leguminosae. Their findings on macroscopically similar growth abnormalities differ greatly. Observing polyploidy in the majority of tumor cells in sugar-beet crown gall tissue, Winge supported the hypothesis that tumors result from somatic polyploidy. Wipf also noted polyploid cells in her study of root nodules of leguminous plants caused by *Rhizobium*, although some authors deny these root nodules a place among real plant tumors. Whitaker, however, did not find a single polyploid cell in his material. Mitosis was very regular in *Nicotiana* tumors and the few irregularities found were not considered significant. In normal stem tissues there were as many as four nucleoli within the nucleus. In tumor tissue most nuclei contained two nucleoli but their number varied within the limits for normal tissue. While these three tumors were macroscopically similar in certain respects, their cytological differences were great. Thus, although polyploidy or some other cellular phenomena were encountered in some cases, there seemed to be no common factor accountable for the formation of these overgrowths. It was hoped that in the present study it would be possible to produce some form of crown gall in which the cytological phenomena involving polyploidy might be accurately observed.

The problem of the study of plant overgrowths is complicated by the factor of specificity, or perhaps it should be expressed as a lack of specificity. It has been very commonly observed that *Allium cepa* bulbs which have been carelessly treated and mutilated by deep cuts in order to obtain a greater production of roots, manifest more or less irregularity in growth. With this in mind the author attempted to study briefly the specificity of action of *Bacterium tumefaciens* on *A. cepa* as compared with such agents as *Escherichia coli*, *Bacillus subtilis*, a synthetic hormone (diethylstilbestrol), and an auxin (indoleacetic acid), as well as chemical and mechanical injury. Among these agents, diethylstilbestrol has been reported by Lettre (17, 18) to inhibit mitosis in much the same manner as colchicine; it was also compared by Zollikofer (42) with plant hormones such as indoleacetic acid. It therefore seemed very desirable to study its effects on *A. cepa* since abnormal growth, often in the form of a tumor,

sometimes results from treatment with substances which possess a colchicine-like action.

MATERIALS AND METHODS

Small bulbs, as well as seeds, of *Allium cepa,* Yellow Globe variety, were used. Bulbs averaging 35 gm. in weight were selected for roundness and size of stem base; none were used which had an odd or irregular shape. All bulbs were grown in half-pint glass containers. Seeds were germinated in Petri dishes with one layer of filter paper 9 cm. in diameter. Generally some water was added to allow for evaporation except in those cases in which the experimental plants were kept in moist chambers. In some cases, roots from bulbs were treated in small containers of about 10 cc. capacity. Sterile instruments and containers were used wherever possible. All experiments were conducted in moderate light at approximately 24°–26° C. and in a few instances at 30° C.

Bacterium tumefaciens culture 4720 from Georgetown University was propagated by transfers on "Difco" dextrose agar. Onion decoction media, such as onion decoction and 2 per cent onion decoction agar, as described by Anderson (1) for a typical *Allium cepa* pathogen, *Urocystis cepulae,* were used either in their natural condition with the normal pH of 5.5 or titrated to pH 7.0. Cultures were grown at approximately 24°–26° C.

The effects produced by *Bacterium tumefaciens* were studied on growing roots from bulbs, on germinating seeds, young plants and bulbs, and in particular on the stems of bulbs. Roots from bulbs were grown directly in the bacterial culture on onion decoction medium or in tap water to which bacteria were added daily. Germinating seeds and young seedlings were brought in contact with the bacteria by introducing the bacteria into water used for moistening filter paper in the Petri dish. In other cases the epidermis of growing roots and young seedlings was scraped with a bacteria-laden needle; or an aqueous suspension of bacteria was introduced by means of a thin gauge needle into growing roots and young seedlings. Bulbs were inoculated with *B. tumefaciens* by various methods: particularly in the preliminary experiments, the injection of aqueous suspensions of bacteria in various regions of the bulb such as the growing green leaves, in and between the scales, and in the base of the stem; spreading of bacteria over a freshly scraped stem; growing the plants in the bacterial culture; pricking the center of the stem with a needle carrying bacteria; and transfers from previously produced infections. This last method was performed variously by applying infected tissue to stems of healthy plants, by injecting juice extracted from infected bulbs, and by reinoculation with bacterial cultures isolated from infected bulbs.

Bacteria were isolated from bulbs of *Allium cepa* in each case of infection. Gram stained smears were prepared and the general features of each isolated culture recorded for comparison with the original culture

used as inoculum. Media in which infected plants were grown were checked for the presence of *Bacterium tumefaciens*. The effects produced by the bacteria in respect to changes in hydrogen-ion concentration were studied in the case of infected plants and onion decoction media.

Various other agents were used, such as needle-prick inoculations of *Escherichia coli* and *Bacillus subtilis*, diethylstilbestrol in various concentrations ranging from 0.0025 per cent to 1 per cent solutions, a solution of 0.2 per cent indoleacetic acid, a solution of 1 per cent chloral hydrate, solutions of 0.5 per cent and 0.25 per cent podophyllin, and an injection of 0.025 cc. of glacial acetic acid. The effects of diethylstilbestrol (Merck & Co.) were studied on the germination and the development of seedlings, on roots growing from bulbs, and on excised root tips as well as on the stems of fully grown bulbs. These results were reported elsewhere (14). Deep cuts by which most of the stem was removed from the bulb were made to study the effects of mutilation. Treated and control material was examined cytologically at frequent intervals.

The cytological technique consisted of the following: fixation with 1:3 acetic-alcohol for smears and with Craf for sections; staining with Feulgen with or without fast green counterstain and with aceto-orcein for smears; iodine gentian violet, Feulgen, and iron-alum haematoxylin for sections cut at 10 micra. Gram stain was used for bacteriological smears.

MACROSCOPICAL AND BACTERIOLOGICAL OBSERVATIONS ON MATERIAL TREATED WITH BACTERIUM TUMEFACIENS

Roots from bulbs growing in tap water to which *Bacterium tumefaciens* was added daily showed no external effects of the treatment during one week. A yellowish discoloration, particularly in the root cap region, of some of the roots grown directly in cultures of the bacteria in an onion decoction at pH 5.5 or pH 7.0, with clear evidence of wilting in a few cases, was most probably not caused by the bacteria because the sterile media produced similar effects. Attempts to introduce bacteria into the roots by scraping roots with a needle or injecting minute amounts of an aqueous bacterial suspension resulted in only occasional side roots at or near the point of injury. It was impossible to infect bulbs by needle prick of the roots or by placing the roots in a bacterial suspension.

The treatment of seeds with water suspensions of bacteria did not impair their power of germination. Seedlings grown in the presence of bacteria had no visible signs of infection and all attempts to produce such signs by pricking the young seedling either before or after the appearance of chlorophyll were unsuccessful. The young seedlings treated with *Bacterium tumefaciens* were not raised to maturity and thus it is impossible to establish whether this stage in the life of the onion plant is in reality immune from *B. tumefaciens*. Izrailskij (13) obtained 20–30 per cent infection by treating seeds of *Beta vulgaris* with a culture of *B. tume-*

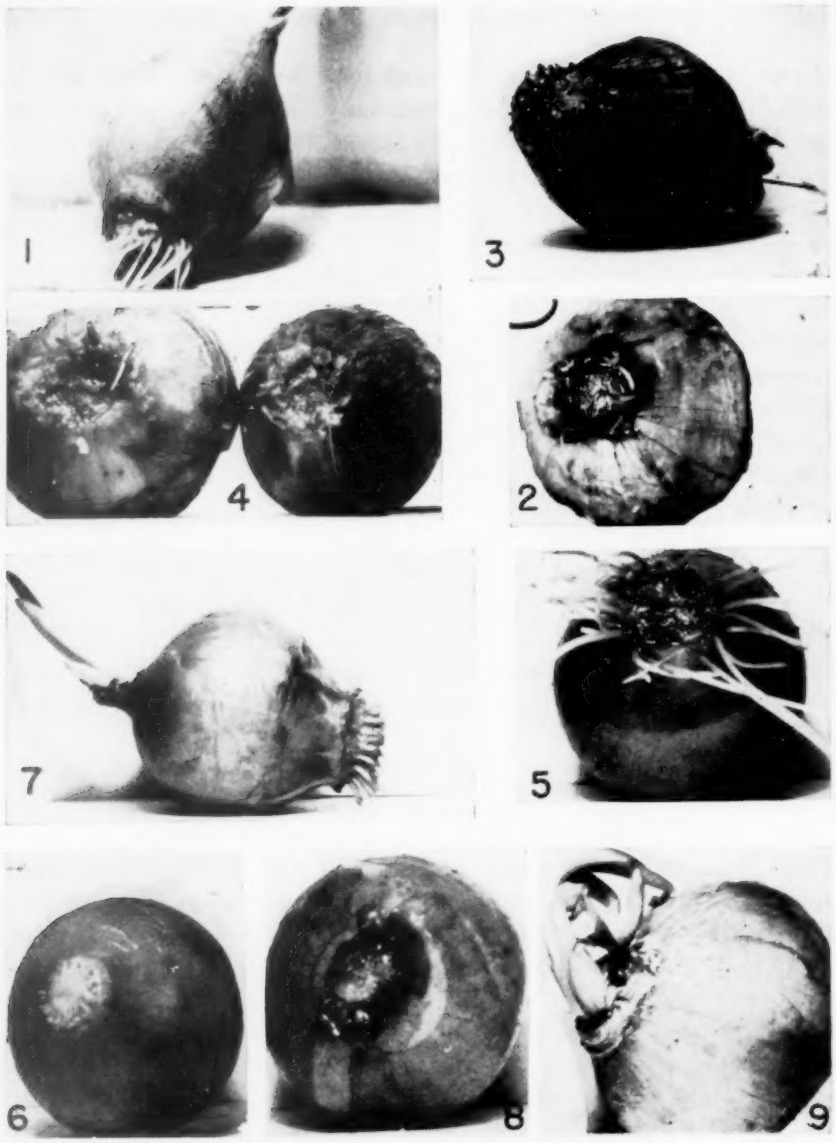
faciens. It may, however, be supposed that the bacteria responsible for this 20–30 per cent infection did not actually infect the germinating seeds but, by surviving near the seedlings, infected them later whenever a wound provided a portal of entry. This seems plausible in view of Izrailskij's report that 100 per cent infection followed the pricking of young plants, with consequent introduction of bacteria.

Various methods of inoculation were employed in preliminary experiments to ascertain the susceptibility of *Allium cepa* bulbs to *Bacterium tumefaciens*. A true case of infection generally followed inoculation involving the base of the stem. However, the time necessary for manifestation of symptoms was variable. The most successful method of inoculation, employed throughout the experiments as the principal means of infection, was by needle prick into the center of the stem base. No visible effects were obtained when the bacteria were introduced into the upper portion of the bulb, which consists of scaly leaves only. Needle-prick inoculation has produced infection in more than 30 bulbs (Figs. 1–3). However, an approximately equal number of bulbs did not have the expected symptoms of infection after needle-prick inoculation. This could be explained by changes in virulence of *B. tumefaciens* cultures. Such changes were reported by early investigators and confirmed by Levine (25) and others.

The first appearance and progress of the infection was easily observed. As early as two days after needle-prick inoculation, a slight swelling could be detected at the base of the bulb just above the margin of the scale leaves. Some control bulbs also had a slight swelling detectable only by palpation, but this swelling never developed further into a localized abnormal overgrowth as in the case of bulbs infected by *Bacterium tumefaciens*. The bulbs remained apparently healthy except for the widening of the stem base along one diameter and a loosening of the external scales during the early stage of infection, usually within a week after inoculation. In some cases the widening of the stem was accompanied by a local break in several layers of scales and an enlargement of the root-bearing area.

A typical abnormality which usually developed in about 13 days, or in some plants 8 days, consisted of a horizontal elongation of the stem base. The outer scales at one end of this elongation were broken and a white bulbous swelling, suggesting a kind of tumor, appeared from beneath. This tumor or overgrowth was composed of the inner scales which manifest a degree of curvature different from that of the other scales. The loss of symmetry was very evident. The base of the stem in the controls maintained its circular shape and reached the approximate size of 16 mm. $\times$ 16 mm., compared to 20 mm. $\times$ 16 mm. in infected plants.

This outpushing of inner scales occurred gradually and after reaching a definite size, within approximately 2 weeks, did not progress further. From this time on degenerative changes took place in the outer portion of



FIGS. 1 to 9. Bulbs of *Allium cepa* about ¾ normal size.

(1) Seven days after needle-prick inoculation with *Bacterium tumefaciens*. Widening of stem base and loosening of outer scales.

(2) Twenty-two days after needle-prick inoculation. Spreading of stem, bulbous projection at one side of stem, and advancing degeneration of tissues. Roots have been removed.

(3) One month after needle-prick inoculation. Roots have been removed and plant is somewhat dried. Center of stem shows area injured by inoculation.

(4) Application of tissue from a diseased plant to a healthy bulb results within 14 days in destruction of tissue in the new host.

(5) Spreading of stem 18 days after application of tissue from a diseased plant.

(6) Plant treated for 13 days with 1 per cent solution of chloral hydrate after 24-hr. recovery in tap water. Roots and leaves suppressed; base of bulb slightly swollen.

(7) Plant treated with 0.2 per cent indoleacetic acid in the form of lanolin paste applied to needle-prick wound in center of stem. Stem vertically elongated.

(8) Injection of 0.025 ml. of glacial acetic acid in center of stem base resulted within 11 days in severe injury with necrosis spreading in all directions.

(9) A deep cut in the base of the stem caused abnormal growth of leaves downwards through cut surface.

the bulb, involving the stem and the lower portion of the scales. Frequently the center of the stem showed injured regions where the bulbs were inoculated. After the roots had been cut at this stage no new roots appeared, because of the degeneration of root primordia.

The inoculation of *Allium cepa* bulbs with *Bacterium tumefaciens* resulted in a disease which caused premature death of the plant. Before decay was complete, the bulbs showed evident signs of wilting which could be attributed to the fact that all the vessels supplying the upper portion of the leaves had been destroyed together with the other tissues. Dying plants often were attacked by molds such as *Rhizopus* and a pink mold, probably *Neurospora*. The described effects were never obtained after similar inoculation on plants growing in water at 30° C. Eradication of the diseased area even as late as the 20th day after inoculation preserved the life of the plant. This seemed to indicate that the bacteria were limited in most cases to the regions adjacent to the point of inoculation injury and, moreover, the bacteria were concentrated near the necrotic tissues at the surface. This would be in accord with the aerobic habit of *B. tumefaciens* and its normal behavior in the host, as noticed by early investigators (33). Plating out of the tissues from diseased plants yielded colonies of *B. tumefaciens* with milky-white coloring, gumlike consistency, and entire borders on dextrose agar. Similar cultures were obtained when the fluid from the lower part of the diseased bulb was drawn out with a sterile syringe and plated.

The production of infection in healthy bulbs by means of tissue taken from infected bulbs was successful in 15 instances. Identical characteristics of the original disease were produced by direct application of a piece of diseased tissue to a healthy plant (Figs. 4, 5), by injection of sap of the diseased plant, and by use of cultures isolated from infected plants. By this last procedure in which *Bacterium tumefaciens* isolated from an infected onion bulb was used to produce a new infection, the etiological agent was ascertained according to Koch's postulates. From all these cases of infection a Gram negative bacillus, similar to that of the original culture, has been isolated. In some instances the shape of the bacteria isolated from the plant differed slightly from the original culture, being shorter and sporelike in appearance, but, on culturing in agar medium, the bacterium recovered its original characteristics. This observation seems to be another instance of the morphological changes in *B. tumefaciens* which have been pointed out by early investigators such as Levine (22, 25).

The effects of infection by bringing a piece of diseased tissue in contact with a healthy stem were seen as a widening of the stem base and a bulging which caused breaking of the outer scale covering. Fourteen days after application, degeneration of the tissues was apparent. It seems that the bacteria passed from the diseased tissue to the healthy tissues of the stem, which represented a more adequate substrate. Their access was

facilitated by the fact that in scraping off the old roots some regions were inevitably injured. The crushed cells from this small area or areas of injury provided an entrance into the new host, while at the same time the bacteria were harbored in relatively large numbers very close to the stem in the infected necrotic tissue that had been applied to it.

All of these cases of infection by *Bacterium tumefaciens* seemed to indicate that *Allium cepa* was susceptible to this form of crown-gall disease when fully grown bulbs were raised in tap water. This appeared to be contrary to the expectation of Smith and Quirk (36) based on the pH range which was believed to be tolerated by *B. tumefaciens*, although Berridge (3) considered pH 5.2 in the plant sap as most favorable to growth of *B. tumefaciens*. The case of *A. cepa* is not an isolated one, since many of the plants supposed by Smith to be naturally immune from *B. tumefaciens* have been proven susceptible to this organism when it is artificially inoculated.

The pH of *Allium cepa* sap as measured by the author was between 5.0 and 5.5. The pH of the sap in infected plants was between 6.5 and 7.0. This was in agreement with the findings of other investigators who also observed that galls produced by *Bacterium tumefaciens* were more alkaline than normal tissue of the host plant (7, 15, 16). Thus the pH of the onion juice did not noticeably inhibit in any way the action of the bacteria, especially when the bacteria were placed on a tissue which was in part bruised by the inoculation. In experiments with *B. tumefaciens in vitro*, it was found that the bacteria had the ability to change the pH of onion decoction medium from 5.5 to 6.5 in 48 hr. at room temperature (24°–26° C.), while the pH of the sterile decoction remained 5.5.

The effects of *Bacterium tumefaciens* on *Allium cepa* bulbs are not similar to what is commonly known as crown gall. It was not a wartlike growth with a rather dry covering; it did not resemble closely the other types of crown gall such as the leafy crown gall, the wooly knot type, and the fasciation type. Most naturally occurring crown galls depend on the vessels of the plant for water, but this supply is limited when constriction occurs at the point of junction between the gall and the stem. Such a gall has its period of growth, development, and senescence, as well as death, and all these can occur independently of the host (21). However, the water supply of the onion overgrowths, composed of scales and stem base, was practically unlimited since the bulbs were partially immersed in water. Moreover, in the *A. cepa* overgrowths, the infected portion was an integral part of the plant and the fate of the overgrowth and the infected bulb was identical.

Dried infected bulbs kept at 100° C. for several hours a day for more than 20 days were used to inoculate other healthy plants, but no effects were produced by the application of such dried diseased tissue. Evidently the bacteria which were the agents responsible for the described effects were completely destroyed by heat. The thermal death point of *Bacterium*

tumefaciens was described in the literature as 50° to 51° C. for 10 minutes. Even lower temperatures of 46° and 47° were considered injurious when applied for prolonged periods (4).

The bacteriological observations seemed to definitely attest to the presence of *Bacterium tumefaciens* in the infected plants. Short Gram negative rods similar to those of the original culture of *B. tumefaciens* have been isolated in each case of infection. The stem surface plating of the infected plant on agar media revealed the presence of Gram negative bacteria believed to be *B. tumefaciens,* as well as some longer and more slender Gram positive rods similar to those found in tap water. The fluid drawn out of the infected plants near the stem base contained cell debris and Gram negative bacteria, as well as the mentioned Gram positive rods. The tissue of dying malformations on the infected plants about 38 days after inoculation, smeared by ordinary bacteriological methods, showed Gram negative rods among the cell debris. Sap drawn from the portion of the bulb above the stem and between the scales contained Gram negative bacteria. Numerous Gram negative bacteria were obtained from the water in which an infected plant had been growing for 36 days. Thus, by bacteriological methods it could be established that the bacteria inhabit the decaying tissues and are found at the surface rather than within the diseased stem. This surface position renders it an easy matter for the bacteria to fall into the surrounding water whenever the decaying tissue breaks away. The bacteria also are found in the sap above the stem and in the stem portion that becomes rather soft as infection progresses. *B. tumefaciens* was not observed, however, either in Feulgen smears of the infected tissue or in paraffin sections stained with either iodine gentian violet or Feulgen. Many authors, among whom are Robinson and Walkden (33), were unable to see the bacteria in the tissues although they could ascertain their presence by plating out the diseased tissues. Whenever the bacteria were seen within the cells, which was reported as only occasional by Riker (29) and Magrou (27), it was doubtful whether the small bodies seen within the cells were the bacteria or some cytoplasmic constituents of the cells. For this reason, Milovidov (28) proposed a rather complicated procedure for the differential staining of bacteria and mitochondria, while Wright and Skoric (41) suggested the use of Giemsa stain with a pre-treatment with a buffer solution. The latter methods are of great value in the study of root nodule bacteria in Leguminosae but were not employed in this investigation. More recently, Brown and Evans (6) claim to have observed the bacteria in cortical cells of the roots, in tracheids, and in medullary ray fibers of conifers infected by *B. tumefaciens,* but they do not mention the method used. In *Allium cepa* the only indication of bacteria are strands of darkly staining tissue, apparently necrotic, which infiltrate among the normal tissues and seem to be identical with what Robinson and Walkden (33) referred to as "zoogleal masses."

MICROSCOPICAL OBSERVATIONS ON MATERIAL TREATED WITH BACTERIUM TUMEFACIENS

Detailed observations of roots and seedlings treated with *Bacterium tumefaciens* yielded no result of cytological importance. Evidently the bacteria never penetrated the roots, and no sign of injury could be detected. Even in roots and seedlings which had been pricked, there was only limited development of side roots. The regions in which the side roots arose showed, as was expected, a large number of perfectly normal mitotic divisions. It may be concluded that the presence of bacteria did not disturb the development of roots and seedlings.

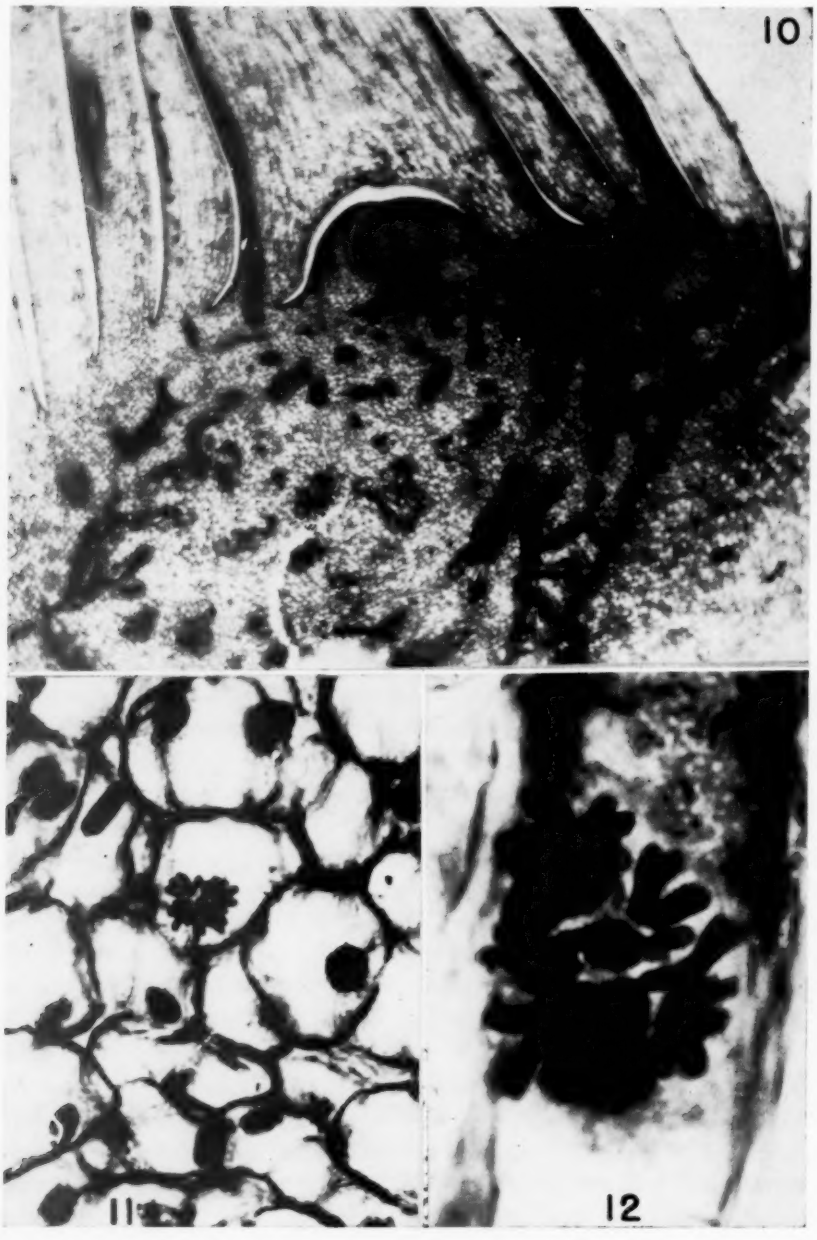
Roots growing from bulbs in the onion decoction (at pH 5.5 or 7.0) which had been heavily inoculated with *Bacterium tumefaciens* were not significantly affected by the bacteria. The number of mitotic divisions in the meristem appeared normal, with all stages of mitosis present. The same material was studied in sections since it was necessary to make certain whether or not any effects were produced on the regions of elongation. This effect would be expected if some auxin-like action were involved, since it was demonstrated (2) that divisions may be obtained in the region of elongation by the application of naphthalene acetic acid. However, divisions were not observed in either the region of elongation or region of differentiation. Aceto-orcein smears were made daily throughout one week in order to study the cells of stems taken from bulbs which had been inoculated by needle prick, but they did not yield significant observations.

The best preparations for the study of position and relationship of cells were sections cut at 10 micra and stained with iodine gentian violet. Six days after the inoculation, the normal morphology of the stem was in no way altered except for the point of inoculation. It was possible to distinguish the definite pith and cortical parenchyma as well as numerous root primordia at the periphery of the stem. The vascular bundles appeared rather regularly scattered throughout the cortical and pith parenchyma. In close proximity of the needle-prick inoculation point, a region of cells which stained deeply, the cell walls were colored a deep blue with gentian violet. These cells were often devoid of nuclei, or some nuclei appeared notched and crenated and simulated some of the so-called amitotic fission stages to which early investigators (35) attached great importance. These features, however, were not characteristic inasmuch as they generally can be found in most injured tissue. Of course, their number in this early stage was very limited but increased as the infection progressed.

When bulbs were grown directly in the bacterial culture rather than in tap water, it was possible to notice a path of destruction very probably followed by bacteria, although the bacteria could not be demonstrated by staining. The portions between the root primordia seemed to become involved in the early stages, and destruction of the tissue proceeded toward the vessels. The destroyed tissues appeared to block the usual path of the

flow of water to the upper regions of the plant. Intercellular spaces left by the collapsed dead cells were also observed at this stage. Above the root-bearing area, the region between the basal portions of the scales was subject to destruction by the bacteria or by the products of the bacterial culture, particularly the cells along the margin of such scales, and the parenchyma cells. The effects of inoculation by needle prick, however, were more limited and localized. Even 28 days after inoculation the periphery of the stem remained fairly healthy, while the region around the point of inoculation was destroyed. The destructive effects were more evident in the outer scales in which at this time growth stopped and a slow wilt and decay began.

The amount of decaying tissue in the stem proper varied from plant to plant. Thus the sequence of the histological features discussed below does not necessarily represent the sequence of events during *Bacterium tumefaciens* infection. The sequence was as follows: 6-day-old cross section, 10-day-old longitudinal section, 15-day-old cross section, 19-day-old cross section, 21-day-old cross section, 28-day-old cross section, and 32-day-old longitudinal section. It is important to remember that the response to treatment of the same set of experimental plants occurs at varying times; thus the typical overgrowth composed of a spread stem and excessive curvature of scales occurred on the average after 13 days, but in some plants after 8, 12, 14, and 19 days. The material for microscopical observations was chosen according to the general macroscopical response which was common to most bulbs at definite times. In the early stages of the disease it was easy to preserve entire stems with several attached scales, but as the disease progressed it was frequently impossible to fix the disintegrating scales. Hence, in the later stages it was necessary to use material from the stem alone and in particular from the portion nearest the overgrowth. It was believed that this portion would reflect the true condition of the plant. Few general statements can be made in regard to this material. True disintegration of tissues involving large areas did not appear before the 15th day after inoculation (Fig. 13). There were no divisions in the stem tissue although this tissue clearly was spreading. Divisions were seen occasionally in leaf and root meristems and in pericycle cells in the differentiated regions of roots. There were many more cells in 15-day material than in 6-day material. Cells were small but no division in the 15-day material could be seen. In general, none of the material except that treated for 10 days with bacterial culture had dividing cells. This lack was understandable in the later stages when a general degeneration of tissues set in, but some dividing cells might have been expected during the early stages when size of stem and of basal portion of scales was increasing. Several factors, however, can also be considered responsible for the early macroscopical changes, such as the increase in the root-bearing area due to observed activity of the pericycle cells, and the formation of intercellular spaces which by passive intake of water may increase the size of the bulb.



FIGS. 10 to 12. Effects of a 10-day treatment of *Allium cepa* bulbs in a liquid culture of *Bacterium tumefaciens*. Longitudinal sections, stained with Iodine-Gentian Violet.

(10) Upper portion of stem and basal portions of scales. A polyploid cell was observed in basal region of central leaf meristem which stands out as the involuted structure in center of field. (×24)

(11) Dividing cell in cortical parenchyma of stem below central leaf meristem. (×768)

(12) Polyploid cell from region shown in figure 10, containing tetrachromosomes. (×1568)

Divisions first were observed in the cortical parenchyma in 10-day-old material which had been treated with bacterial culture (Figs. 10–12). In the cortex of leaves there were definitely elongated cells which contained very small amounts of cytoplasm but were in division. The most striking feature of this tissue, however, was the variable size of nuclei and chromosomes in adjacent cells of approximately the same dimensions. There was some indication of polyploidy, although an accurate chromosomal count was extremely difficult. A typical polyploid cell with some tetrachromosomes was observed in another region among cortical cells near the basal region of the central leaf meristem (Fig. 12). The tips of the tetrachromosomes showed a characteristic separation which indicated their doubleness. The size of the chromosomes and of the metaphase plate were definitely greater than that of ordinary diploid cells. A careful count gave 28 + chromosomes. The occurrence of polyploid cells in this material has not been frequent enough to indicate that polyploidy is an essential feature of *Bacterium tumefaciens* infection. It was impossible to establish polyploidy on the basis of number of nucleoli, since most of the cortical cells having very large nuclei contained two rather large or even a single unusually large nucleolus. The occurrence of polyploid cells in division, limited apparently to this material which had been treated with *B. tumefaciens* culture for 10 days, was the only instance in this investigation in which polyploidy could be definitely established by chromosomal count. In the normal material grown in tap water no such polyploid divisions were observed, but many cells of the cortical parenchyma appeared as large as the polyploid cells actually found in the treated material. This treated material had been grown in an onion decoction medium in which *B. tumefaciens* was growing and to which the bacteria added their metabolic products during 10 days. This combination of factors may have had a stimulating effect on the polypoid cells and thus revealed their nature, since a sterile onion decoction alone did not produce this effect. The same is true of *B. tumefaciens* when applied alone by needle-prick inoculation. Thus, if the divisions obtained in this case were to be considered as effects of the combination of factors, it should be suggested that this combination of factors did not produce but helped to reveal pre-existing polyploidy. It is possible that in the adult onion bulb great numbers of cells in the cortex are in the polyploid condition but do not undergo division.

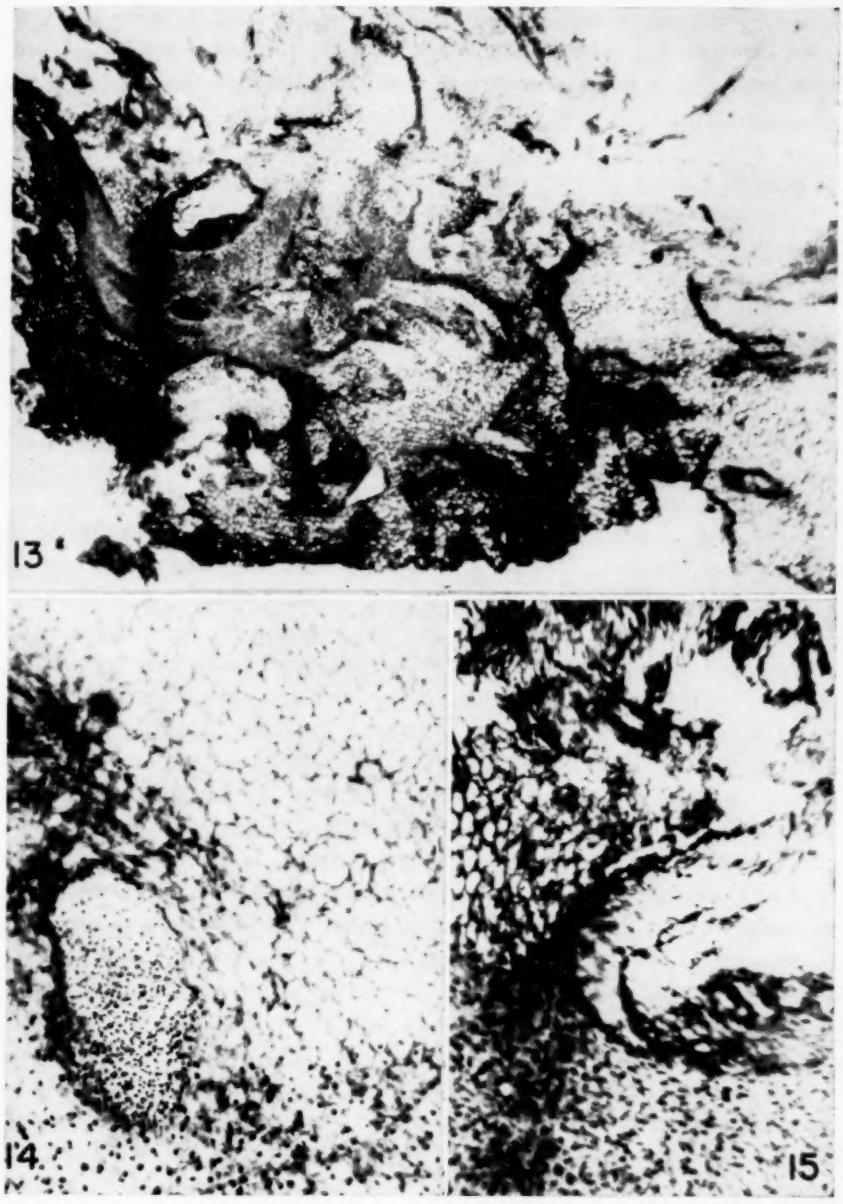
Older stages of the disease were studied first since it was necessary to ascertain the outcome of inoculations. The macroscopical observations were carried on in some cases until the plant died; in others, the plant was fixed after the appearance of the most drastic symptoms of disease, such as general wilting and beginnings of decay, but in time to obtain tissue which could withstand handling. In the study of the earlier stages the early symptoms were noticed and it was inferred that the inoculation had resulted in infection and the plant could be expected to develop other symptoms later. It was of great disadvantage that material from the same plant

could not be collected at various time intervals. Excision of the infected stem tissue effected a cure of the plant, especially when the bacteria were localized near the point of inoculation. Thus the considerations and comparisons between the material of 2 and 6 days after inoculation are not significant, since they refer to material that was taken from different bulbs, although the bulbs were undergoing identical treatment. However, when longer time intervals were allowed for greater changes to take place, the results could be considered as roughly representative of stages of the disease.

When 6-day-old material was compared with 15-day-old material, the most striking feature in the older material was a disorganization of all tissues (Figs. 13–15). The decay manifested itself mostly at the periphery of the base of the bulb in the regions occupied ordinarily by the bases of the scales. The stem proper in cross section revealed a destruction of root primordia, the most outstanding feature being a strandlike formation of parenchyma cells, very numerous and compact, which appeared to infiltrate the stem periphery.

As the degeneration of tissues advanced, more and more intercellular spaces were formed. Root centers were definitely destroyed. Some nuclei were highly pyenotic, others vacuolated. No mitotic divisions were present. Many parenchyma cells were devoid of nuclei and cytoplasm. Frequently cells of the vascular bundles had notched and crescent-shaped nuclei. Extranuclear chromatic granules were numerous. Formation of such nuclear fragments in the cytoplasm has been observed by many authors on crown gall material. Related phenomena characteristic of cellular and nuclear degeneration have likewise been reported. In all these advanced stages the vascular bundles were less numerous than in healthy stems. The great relative increase in cortical parenchyma cells may be a source of error in estimating the number of vascular bundles. It may be that the formation of new bundles, as it takes place in a monocotyledonous stem, may have been prevented in some way by the infection.

The main features accompanying the infection by *Bacterium tumefaciens* as seen in these microscopical preparations can be related to the macroscopical progress of the infection. The large increase in the number of cortical parenchyma cells, so characteristic in 15-day preparations and verified by the divisions observed in 10-day treated material, roughly coincides with the period of development of the abnormal growths on the bulb. The increase in the root-bearing area is correlated with the divisions occasionally seen in pericycle cells of the differentiated regions of the root which lead to the formation of new roots. The formation of intercellular spaces in the early stages of infection by destruction of cells and the consequent imbibition of water seem to be related to the bulging of the scales. The final necrosis of a large part of the stem with the eventual wilting and death of the plant is undoubtedly related to the destruction of vascular elements of the stem and destruction of the cells which become devoid of



FIGS. 13 to 15. Late stages of infection produced on *Allium cepa* by needle-prick inoculation with *Bacterium tumefaciens*.

(13) Destructive changes observed in cross-section of stem 15 days after needle-prick inoculation. Iodine-Gentian Violet. (×24)

(14) Areas of pyenotic nuclei and of dead parenchyma cells near a degenerated root primordium in the same plant. (×80)

(15) Another region in the same plant showing advanced degeneration of tissue and numerous intercellular spaces. The root primordium is completely destroyed. (×80)

nuclei and cytoplasm and finally collapse. Polyploidy as seen in 10-day treated material is limited to the cortex near the leaf meristem and possibly the leaves, but it is not a pronounced feature of the infection.

DISCUSSION

Previous studies of crown gall have been made almost exclusively on dicotyledonous plants. The infection of *Allium cepa* by *Bacterium tumefaciens* is in this respect unusual. The bulbs raised in tap water in the laboratory appeared actually infected and not merely carriers of the bacteria which were later isolated from them. No infection was produced by *Escherichia coli* or *Bacillus subtilis*, both of which are nonpathogenic to plant and animal. The effects produced by *B. tumefaciens* were characteristic in their gross appearance and could not be considered identical with those produced by other agents, although it is not excluded that other agents which would have provided the same type and amount of stimulation as did the bacteria might have produced similar effects.

Crown gall is ordinarily a disease of the stem but other parts of the plant may be attacked. The stem in *Allium cepa*, the part which responds promptly and visibly to the bacterial infection, differs greatly from the usually erect stem of a flowering plant. It is very limited in size and it is overshadowed by the large mass of fleshy scale leaves as well as by the large number of adventitious roots which arise from the base of the bulb. The stem of the onion bulb which was inoculated in these experiments was immersed in water. The resulting effects, which differed in a way from the commonly known crown gall, could be attributed to the morphology of the *A. cepa* bulb and to the method of growing the inoculated bulbs.

The response of *Allium cepa* to *Bacterium tumefaciens* at first sight appeared atypical, but it is important to remember that the essential feature of crown gall disease is the causal relation of some macroscopic localized manifestations of disturbed growth with the presence of *B. tumefaciens*. It is well known that crown gall may take a variety of forms. It may be covered or naked, irregular or regular, soft or hard. The gall is not necessarily on the crown of the plant; it may even be on the roots. The gall may readily decay, especially on soft plants, and wilting is one of the accompanying features (5). A wilt-inducing factor has been recently isolated from *B. tumefaciens* (12). On the other hand there are many galls on plants which can easily be mistaken for crown galls, such as knots or galls of the olive tree which are caused by *Phytomonas savastanoi*, nematode galls on yellow flax, and wooly galls due to aphids. Oleander galls caused by *Phytomonas tonelliana* are also very similar to crown galls. All these galls, while they resemble crown gall, lack the essential characteristic of the causal relationship to the presence of *B. tumefaciens*.

The overgrowth in *Allium cepa* resulting from inoculation with *Bacterium tumefaciens* was a localized disturbance involving the lower portion of the scales and the base of the stem and was caused by the *B. tumefaciens*.

Sterile needle prick experiments did not produce overgrowths of any kind. In addition, no results were observed when diseased tissue, which had been dried by heat, was applied to healthy plants. Finally, the plant could be restored to health when the regions near the point of inoculation were eradicated at an early stage of the infection.

The infection produced on *Allium cepa* bulbs by *Bacterium tumefaciens* had definite constant features. There was a proliferation of the stem in a horizontal direction and an increase in the root-bearing area, although *B. tumefaciens* did not act directly on the healthy roots. There also was a definite curvature of the leaves at their base in a limited region of the bulb. The pH of the sap of infected plants was less acid than that of the normal plant grown in water for the same length of time. Decay was a characteristic feature.

Bacterium tumefaciens in *Allium cepa* was primarily destructive in its effects and it was possible that the abnormal growth of stem and leaves was a secondary effect. This abnormal growth could be considered as a response to bacterial stimulus but it could also be a defensive reaction by which the plant attempts to develop away from the point of injury. This could be equally true of chemical and physical agents. The parallel between the effects of bacterial stimulation and of a strong acid, glacial acetic, is somewhat striking. The acid was applied in minute quantities to the stem by injection and not by needle-prick inoculation. The subsequent spreading of the stem away from the point of injury in the latter case was similar to the reaction in the case of infection by *B. tumefaciens* (Fig. 8). *B. tumefuciens* was able, however, to produce not only a response which could be interpreted as a defensive reaction, but also a typical growth response expressed in curvature of the leaves.

The histological observations suggested that the bacteria were extracellular rather than intracellular. By their presence the bacteria apparently caused a higher concentration of substances outside the cells and thus produced an osmotic flow from the cell interior which resulted in collapse of the cell walls. If the bacteria were within the cells and there elaborated products of their metabolism, the opposite situation may have occurred. Yet the characteristic feature of older stages of infection is the presence of collapsed and compressed cells. From micrurgical studies of Hildebrand (11) there is some evidence in favor of the idea that the bacteria causing crown gall do not live within the living cells.

Although the microscopical and macroscopical observations were definitely correlated, it was still a point of discussion whether there was correlation between the bacterial action and chromosomal phenomena, and whether the bacterial action could be considered colchicine-like, thus leading to polyploidy, or auxin-like, thus stimulating divisions in the region of elongation. Havas (10) considered the action of *Bacterium tumefaciens* as colchicine-like. While it was impossible to find any results which would indicate that *B. tumefaciens* was able to influence cells in division by inhibi-

tion of spindle, even by using heavy bacterial suspensions of *B. tumefaciens* on growing root tips, there seemed to be an indication that *B. tumefaciens* produced some kind of growth response with consequent stimulation of cell divisions in the elongated and extremely vacuolated cells. It also appeared that there was a stimulation of divisions of the polyploid cells of the cortex since these cells are not observed to divide in normal material. There is, however, controversial evidence as to the auxin content of crown gall tissues as well as to the amount of beta-indole-acetic acid produced by *B. tumefaciens in vitro* as compared with the quantities necessary for the production of overgrowths similar to those produced on plants by *B. tumefaciens*. Link and Eggers (26), who found that more auxin is present in hypocotyls inoculated with *B. tumefaciens* than in healthy hypocotyls, believed that disturbance in auxin relationships in infected plants played a role in gall development. On the other hand, Riker, Henry, and Duggar (32) could find no significant difference in auxin content of tissues treated by *B. tumefaciens* and the untreated tissues, and they concluded that other factors in addition to auxin were responsible for overgrowths and in particular for the characteristic pathogenicity of *B. tumefaciens*. The mechanism of plant overgrowth production by *B. tumefaciens* still remains undetermined.

The cytological observations on tissue infected with *Bacterium tumefaciens* were also inconclusive. Physical or chemical agents that could be applied to a plant in "pure" form helped eliminate many unknown factors, but the crown gall bacteria as living systems introduced many unknown factors. At the same time it should be noted that no well-defined and certain cytological effects have been reported in the literature. The authors who studied crown gall cellular phenomena often agreed in their description of the degenerative changes such as the breakdown of nuclei, notched and cleft nuclei, as well as furrowed nuclei simulating amitosis. These authors also referred to occasional binucleate cells and to various cell inclusions which they attributed in most cases to the failure of cell wall formation. There seems, however, to be no agreement concerning the role of the chromosomes in the crown gall disease. Riker (30) did not obtain evidence for amitosis, which was one of the favored theories of Smith (34), nor did Riker obtain evidence for multinucleate cells in crown gall tomato tissue. He believed that in consequence of repeated cell divisions nuclear size was reduced. On the other hand, Levine (21) observed in *Beta vulgaris maritima* giant cells with 4n and 8n chromosomal complexes, and attributed the production of higher chromosome numbers to the failure of chromosomes to separate as well as to the failure of cytokinesis to follow karyokinesis. Winge (39) is the investigator who attached utmost importance to the occurrence of polyploidy in sugar-beet tumors produced by *B. tumefaciens*. Winge determined that the majority of cells were polyploid, not only by chromosomal counts but also by the difference in diameters of cell nuclei. From his description of cells found in beet tumors, it seems that Winge

was possibly dealing with a case of polysomaty similar to that found in *Spinacia* (9). The so-called "diakinesis-like" somatic metaphases would indicate the paired metaphase plates as found in the first division immediately following double reduplication in the resting stage. The assertion by Winge that at the time he was entirely familiar with the normal cytology of *Beta vulgaris maritima* and that only diploid cells were found in the root tips studied, could not withstand more recent evidence. Levan (20) reports that in second-year root tips in sugar beets there is normally a chromosomal doubling. The tumors studied by Winge arose during the winter and it can be supposed that in the time required several reduplications of chromosomes in the resting stage may have occurred. Winge was convinced that the polyploid divisions he found in gall tissues were due to the *B. tumefaciens* infection. He was impressed by the mitotic disturbances leading to changes in chromosome number in animal tumor tissues and by the idea that the chromosomal disturbances might be the cause of malignant tumors in general. The frequency of polyploidy in the beet tumors seemed to substantiate his opinion, especially since he was convinced that normal cells in the root tips contained only diploid numbers of chromosomes. Although Levan's results were based on a small number of roots and are probably not sufficient to establish conclusively that polyploidy is in this species a part of the normal development, they indicate that polyploid tissue may occur in sugar beets over a year old. It is possible that in the case of crown gall on beets polyploidy was not caused, but was revealed, by the infection and by the growth-stimulating effects of *B. tumefaciens*, especially in view of the fact that age and cold storage are known to contribute to chromosomal doubling.

Most authors did not refer to meristematic tissues. Even when tissue was described as embryonic, it actually appeared as parenchymatous. The bacteria were localized in either xylem vessels or within dead cells. The problem then arose as to how *B. tumefaciens* could influence the chromosomes in meristematic tissues. This influence would appear impossible unless there were a large number of bacteria in the close vicinity of or within the actively dividing cells. However, it has been proven by many authors that the number of bacteria introduced in a single inoculation did not necessarily affect the size of the gall on the stem. In fact, even single bacteria when introduced into needle punctures of various sizes induced gall formation (11). Nevertheless a single bacterium introduced by Hildebrand into a single living cell failed to produce a gall or to survive. It appears that the bacteria alone do not produce the stimulation necessary for the growth of gall. It is only in combination with a factor such as mechanical injury that the bacteria can infect and cause overgrowth. On *Allium cepa* a needle-prick wound or freshly scraped surface of the stem were prerequisites for infection.

The reaction to infection in *Allium cepa* bulbs was a rather vigorous growth in a region distinct from the region of inoculation. In most crown

galls, true galls arise at the needle prick where *Bacterium tumefaciens* is introduced. It has been suggested by many authors, among them Levine (21), that crown gall is thus a protective reaction of the plant against the invading parasite. A tissue is produced which in the early stages appears uniformly embryonic but becomes characteristically differentiated as it grows older. In *A. cepa* the growth is away from the infected region and the plant does not form tissue to block the infection at the point of inoculation.

Recently there have been many studies in which factors involved in nonbacterial gall formation were investigated. Among these, Flint and Moreland (8), who studied gall formation in decapitated young bean plants, noticed that the healing wound overgrowths which resulted from decapitation developed independently of such gall-inducing practices as the application of indoleacetic acid or of *Bacterium tumefaciens*. On *Allium cepa* the sterile needle prick was not sufficient to produce a growth response.

The use of other agents gave varying results. A very harsh treatment consisting of the injection of a minute quantity of glacial acetic acid in the point where the needle-prick inoculation was ordinarily performed resulted, before the death of the plant, in a spreading of the still-living stem tissue in all directions and a breaking of connection between the injured region and the periphery of the stem (Fig. 8). There was possibly no time for any growth response in the leaves since the very toxic agent caused the early death of the plant. But even in this drastic treatment the effect was fairly distributed around the circumference of the stem. Lanolin paste containing 0.2 per cent indoleacetic acid caused a vertical elongation of the stem which had been as symmetrical as the bulb itself at the beginning of the experiment (Fig. 7). Certain irregular growth responses were obtained with agents other than *Bacterium tumefaciens*. Deep cuts severing great parts of the stem from the bulb produced irregular growth formations at the base of the bulb (Fig. 9). The application of diethylstilbestrol directly to the stem base produced a similar irregular growth response (14). Thus in one case a physical agent and in the other a chemical agent produced a certain deformity. The deformity caused by diethylstilbestrol resembled to a certain degree the response of the bulb to *B. tumefaciens* inasmuch as the stem widened in one of the horizontal diameters. There was, however, no similarity between the diseased condition of the *B. tumefaciens*-treated bulb and the healthy and vigorous appearance of the diethylstilbestrol-treated bulb. It cannot be said at present whether diethylstilbestrol acted in a colchicine-like manner in the production of these plant overgrowths (19).

Although it is difficult to analyze all the factors responsible for overgrowth in *Allium cepa*, it seems necessary to take into consideration the activity of terminal and lateral buds in the interior of the bulb. The experiments seemed to indicate that the deformity which arose on plants which had been deeply cut was due to excessive leaf growth which involved not

only the terminal but also the lateral buds. It is possible that the lateral bud was stimulated in bulbs infected by *Bacterium tumefaciens* and in those treated with diethylstilbestrol. In *Nicotiana* hybrids (37), the greatly increased activity of the lateral axillary buds when the plant reached a certain physiological age resulted in, and seemed to be the main factor in, tumor formation. In the case of overgrowths on *A. cepa* the stimulus of bacteria or of diethylstilbestrol might have upset some regulating mechanism controlling the activity of the lateral bud.

The overgrowth response of *Allium cepa* consists roughly in an increase in number of parenchyma cells. Histologically it resembles the numerous wound overgrowths and chemically produced overgrowths on decapitated plants which were reported during recent years by many authors. Among these are Whiting and Murray (38), who studied the histological response to such agents as nicotine and wounding. In all these cases the overgrowth tissues consisted of parenchymatous cells. In the present investigation it was possible to secure some evidence of divisions among the cortical parenchyma cells in *A. cepa* which had been treated with *Bacterium tumefaciens*. There has been, however, no confirmation that polyploidy is a predominant feature of the crown gall disease. Evidence brought forth in this investigation indicated that the nature of the cortical parenchyma cells which are in a polyploid condition but not destined to divide could be revealed as polyploid by a combination of factors, one of which was *B. tumefaciens*. It seems that some early reports on polyploidy in connection with crown gall might be interpreted in much the same manner.

SUMMARY

Bulbs of *Allium cepa*, when grown in tap water, are susceptible to *Bacterium tumefaciens* introduced by needle-prick inoculation.

An abnormal localized overgrowth of the bulb, which did not closely resemble any of the reported cases of crown gall, has been obtained in 30 cases in about 13 days after inoculation. The application of diseased tissue to healthy plants produced infection in 15 cases. Histological and cytological features of stages of the infection, involving mainly progressive necrosis, have been described. The roots and seedlings of *Allium cepa* were immune from the action of the bacteria.

No confirmation that polyploidy is a predominant feature or factor of crown gall disease was obtained. The evidence suggested that the cortical parenchyma cells of the stem may be in the polyploid condition in the uninfected bulb although not destined to divide. A combination of factors, one of which is *Bacterium tumefaciens*, may reveal polyploidy by stimulation of cell division.

Growth response of *Allium cepa* to indoleacetic acid, podophyllin, glacial acetic acid, diethylstilbestrol, and to partial excision of the stem has been investigated.

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SEED TREATMENT OF FIELD LEGUMES

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INTRODUCTION

The value of chemical seed treatments for numerous types of vegetable seed as a means of protection against damping-off has become well recognized. However, very few definite experimental data have been forthcoming to substantiate the value of such treatment in increasing stands and improving germination of the field legumes, and little is known of the effects of chemical treatment on nodulation.

A common practice of farmers has been to plant legume seed treated only with bacterial inoculum ("Nitragin," *etc.*), no further treatment or protection being thought necessary; but under this practice seeding rates for such crops as alfalfa and clovers have been excessively heavy because of poor stands and the large number of damped-off seedlings. A further belief, fostered by commercial publicity, has been that bacterial inoculum and seed treatment chemicals are incompatible, with the result that growers have avoided chemical treatments. These assumptions did not appear to have a factual basis, and experiments were undertaken at the Oklahoma Agricultural Experiment Station to clarify the problem. The experiments were begun in September, 1946, and continued through July, 1948, under joint sponsorship of the Naugatuck Chemical Division of the United States Rubber Company and the Oklahoma Agricultural Experiment Station.

REVIEW OF LITERATURE

Favorable results have been obtained from seed treatment of alfalfa in Iowa (4), alfalfa and clover in New York (7), alfalfa, lespedeza, and clovers in Pennsylvania (5, 9), and peanuts in several Southern States. Conflicting results have been obtained in tests with clovers in Wisconsin (2) and with soybeans at numerous places (1). General conclusions or recommendations probably should not be drawn from these limited data, particularly since tests have not been made on a number of types of field legumes.

The literature concerning the effects of chemical treatment on nodulation also has been relatively scanty and conflicting. Appleman (3) found

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that satisfactory nodulation was produced upon pea and soybean plants grown from inoculated seed treated with Semesan. Cuprocide, in his tests, prevented nodulation on soybeans and canning peas. Ceresan decreased nodulation on canning peas but not on soybeans. All nodulations appeared to be on lateral roots where chemicals were used, but taproot nodulation was obtained on plants from nontreated, inoculated seed. Kadow, Allison, and Anderson (8) found a decrease of nodulation from an average of 75 nodules per plant (nontreated) to 3 and 4 nodules per plant treated with organic mercury, cuprous oxide, and zinc oxide. Duggar (6) has demonstrated nodules forming on plants from seed treated with Semesan, copper sulfate, saturated boric acid, bichloride of mercury, and concentrated sulfuric acid. Some of the treatments decreased nodulation, while others increased it. Buchholtz (3) reported that small-seeded legumes grown from seed treated with organic mercury dust bore root nodules when planted in the field. Some of the earliest work done by Miller and Stapp, reported by Appleman (3), in Germany demonstrated that if nodule bacteria are in the soil at the time of planting, seed treatment chemicals do not hinder the development of root nodules.

Conflicting reports are found in Litynski's (10) work in Poland, where he observed that some treatments reduced nodulation, and at other times the same treatments had little or no apparent effect on nodulation.

MATERIALS AND METHODS

Certain methods and procedures that were employed throughout the investigations are described here. Variations from the procedures occurred at times, and those deviations will be discussed later. Replicated field and greenhouse experiments were made with Austrian winter pea, mungbean, Chinese red cowpea, yellow hop clover, hairy vetch, and alfalfa. Seed of these were treated with Spergon (tetrachloro-*p*-benzoquinone), Phygon (2,3-dichloro-1,4-napthoquinone), Arasan (tetramethylthiuram disulfide), Dow 9B (zinc trichlorophenate), and Ceresan M (ethylmercuric-*p*-toluene sulfanilide). Nontreated controls, seed inoculated with "Nitragin" bacterial inoculum, and noninoculated seed were included. In all but one of the trials in 1946–1947, the seed were uniformly treated with all the chemical that would adhere to the seed coats; however, in the experiments of 1947–1948 seed was treated at 0.25, 0.50, and 1.00 per cent dosage rates by weight of seed. In the field tests (1946–1947), 5 replications of 200 seed each were used in a randomized block design, for each crop. In the greenhouse the treated seed were inoculated with "Nitragin" and planted in flats or pots. Five replications of 100, 50, or 25 seed, dedepending upon the crop, were planted in a randomized block design for each crop.

In all cases the application of the desired amount of chemical upon the seed was accomplished by shaking seed and chemical together in glass

tumblers. "Nitragin" was used in a dry form and shaken over the treated seed before planting in sterile soil. Soil was sterilized by autoclaving for 3 hr. at 15 lb. pressure.

The average greenhouse temperature for spring and fall was 70° F.; for summer, 85° to 90° F.

EXPERIMENTAL RESULTS

The mean numbers of seedlings produced from treated and nontreated seed for each of the crops tested in the field and greenhouse are given in the summary tables. The mean numbers of plants producing nodules are also included. The analysis of variance for each of the crops was calculated, and appears in summary form. The experimental data for each plot are on file in the Department of Botany and Plant Pathology of the Oklahoma Agricultural Experiment Station.

TABLE 1.—*Effect of seed treatment on the field stand of legumes, Stillwater, Oklahoma, 1946–1947*

Seed treatment	Mean number of plants emerged[a]				
	Austrian winter pea	Mungbean	Chinese red cowpea	Yellow hop clover	Hairy vetch
Spergon	124	89	116	81**	106
Phygon	141	80	104	67**	133
Arasan	153	102	131	40**	163
Dow 9B	151	112	115	51**	146
Ceresan M	116	76	114	25	132
Nontreated	140	97	107	21	149
LSD at 0.05	24	20	.....	12	.....
LSD at 0.01	.....	27	.....	16	.....

[a] Averages for 5 replicates of 200 seeds each.
** Highly significant at 0.01 level.

The Austrian winter pea data are presented in tables 1, 2, and 3. In general, the seed treated with Spergon, Phygon, Arasan, and Dow 9B produced more seedlings than nontreated seed, although not significantly so (Table 1). Plants produced from seed treated with these chemicals also produced more nodules, significantly so with the Arasan treatment. Seed treated with Ceresan M produced fewer seedlings than nontreated seed.

The mean numbers of Austrian winter pea plants produced from seed treated with the different chemicals at varied dosage rates (Table 3) indicate the dosage levels at which the respective chemicals were most effective on this seed. Spergon, Phygon, and Dow 9B were most effective when applied at 1.00 per cent by weight of seed, and Arasan was most effective at 0.50 per cent. The results indicate that the chemicals should not be used in excess of the rates of application indicated.

The mungbean data for 1946–1947 (Table 1) indicate that Spergon, Phygon, Arasan, and Dow 9B were effective chemicals in comparison to the nontreated controls. Further tests conducted with mungbean in

greenhouse trials in 1947–1948 (Table 3) showed all treatments highly significant over nontreatment, with the exception of Spergon at the 0.25 and 0.50 per cent dosage rate. Phygon at 1 per cent gave the highest mean average (33 plants), and in general the plants resulting from seed treated with this chemical were strikingly taller and healthier looking than seedlings from seed treated with the other chemicals. There was some hardening of seed coats with Phygon-treated seed at the highest dosage.

Spergon, Phygon, Arasan, and Dow 9B improved the emergence of cowpea var. Chinese Red in greenhouse tests conducted in 1946 (Table 2) in significantly better germination than nontreatment. Significantly more plants with nodules resulted from these chemical treatments than from nontreatment. The same trend was evident in the field germination trials. In the greenhouse trials in 1948 (Table 3), Phygon, Spergon, Arasan, and Dow 9B at all dosage rates were highly significant over nontreatment.

TABLE 2.—*Effect of seed treatment on stand and nodulation of legumes in the greenhouse, Stillwater, Oklahoma, 1946–1947*

Seed treatment	Number of plants emerging and nodulated[a]				
	Austrian winter pea	Chinese red cowpea	Mungbean	Hairy vetch	Yellow hop clover
Spergon	80–76	87**–80**	80**–78**	74* –71**	85–81**
Phygon	82–77	90**–84**	77**–74**	79**–76**	79–75**
Arasan	87–86*	83**–80**	69**–64**	76* –70**	72–69*
Dow 9B	86–82	78**–75**	71**–66**	82**–79**	68–62
Ceresan M	15–12	51 –49	7 – 4	53 –49	58–53
Nontreated	77–73	57 –52	30 –22	64 –57	59–54
LSD at 0.05	14–11	10 –11	14 –13	9 – 9	8–11
LSD at 0.01	19–15	13 –15	20 –18	12 –12	11–15

[a] Averages for 5 replicates of 100 seeds of Austrian winter pea, Chinese red cowpea, mungbean, and yellow hop clover. The left figure of each pair refers to the number of plants emerged, the right one to the number with nodules.

* Significant at 0.05 level.

** Highly significant at 0.01 level.

Spergon appeared to be very effective on yellow hop clover seed (Table 1). In the field trials, Spergon, Phygon, Arasan, and Dow 9B were each slightly significantly better than either of the other three chemicals. In the greenhouse trial, nontreatment was significantly poorer than treatment with Spergon, Phygon, Arasan, and Dow 9B. Treatment with Spergon was significantly better than with Arasan and Dow 9B. More of the plants produced from the treated seed formed nodules than from the nontreated seed. Nodule formation was lateral, in most cases, on plants which were chemically treated. Spergon was outstanding as to nodule formation on plants resulting from seed treated with this chemical.

The results with hairy vetch (Tables 2, 3) in the greenhouse were promising, but the field data were too variable for confirmation. In one greenhouse test (Table 2) in 1947, seed treated with Spergon, Phygon, Arasan,

and Dow 9B produced significantly more plants than nontreated seed and significantly more of these plants formed nodules. In 1948 a greenhouse experiment (Table 3) revealed that plants produced from Arasan-treated seed were significantly higher in germination than nontreated seed. There was also very evident damage by Dow 9B at all dosages. Spergon at 0.25 dosage rate, and Phygon at the 0.25 dosage rate were significant over nontreatment. The experimental results with alfalfa are clear-cut, as is apparent from tables 3, 4, and 5. Tables 3 and 4 give germination figures.

TABLE 3.—*Effect of seed treatment and dosage on germination of legumes in greenhouse experiments, Stillwater, Oklahoma, 1947–1948*

Chemical	Dosage	Mean number of plants[a]				
		Austrian winter pea	Chinese red cowpea	Mungbean	Hairy vetch	Alfalfa
Spergon	0.00	83	17	6	25	10
	0.25	89*	21**	8	32	27**
	0.50	91**	22**	13	28	33**
	1.00	94**	22**	15*	17	27**
	Excess	81	....	....	....	....
Phygon	0.00	83	17	6	25	10
	0.25	84	21**	23**	32	58**
	0.50	85	20**	32**	31	63**
	1.00	95**	23**	33**	26	61**
	Excess	87	....	....	....	....
Arasan	0.00	83	17	6	25	10
	0.25	83	20**	19**	38	40**
	0.50	94**	20**	20**	49**	40**
	1.00	87*	22**	25**	42**	30**
	Excess	82	....	....	....	....
Dow 9B	0.00	83	17	6	25	10
	0.25	78	21**	19**	5	15
	0.50	83	22**	32**	0	9
	1.00	92**	20**	18**	0	13
	Excess	74	....	....	....	....
LSD between chemicals for whole test						
at 0.05 =		4.00	2.00	8.25	8.0	6.9
at 0.01 =		6.00	2.66	11.04	10.4	9.2

[a] Averages for 5 replicates of 100 seeds each of Austrian winter pea and alfalfa, 50 seeds each of mungbean and hairy vetch, and 25 seeds of Chinese red cowpea.

** Highly significant at 0.01 level.

* Significant at 0.05 level.

Table 5 reports independent experiments investigating nodulation-chemical relationships.

In the first of five replications on November 8, 1947 (Table 3), it was found that Phygon at all dosages, Arasan at all dosages, Dow 9B at 0.25 per cent, and Spergon at all dosages, all were significantly superior to nontreatment. Phygon was the outstanding treatment. There was some damage to seed coats by Dow 9B at the highest dosage rate.

Immediately following the highly significant results of the first test with alfalfa, another five replications were planted on December 15, 1947. Phygon-treated seed was significantly better again at all dosage rates and Arasan-treated seed was significantly better at the highest and lowest dosages. No other chemicals proved significantly superior to nontreatment. One hundred seeds per treatment were planted. On January 5, 1947, another five replications were planted, and once more Phygon showed convincing significance at two dosage rates (0.50 and 1.00 per cent by weight of seed). Arasan was superior with high significance at the 1.00 per cent dosage rate, Dow 9B at the 1.00 dosage being significantly better than nontreatment at the 0.05 LSD level. In this test all plants resulting from Phygon-treated seed appeared to have made a more vigorous growth than those from seed with other treatments. In additional tests with alfalfa an attempt was made to correlate some of the results with nodulation-chemical relationships. Table 4 gives first-recorded germination figures. Phygon-

TABLE 4.—*Effect of seed treatments on alfalfa germination*

Treatment	Dosage rate	Mean number of plants[a]
Check—not inoculated	.....	25.6
Check—inoculated	.....	26.8
Phygon—not inoculated	1.00	36.6**
Phygon—inoculated	1.00	38.4**
Arasan—not inoculated	1.00	30.8
Arasan—inoculated	1.00	30.6
		LSD at 0.05 level = 5.21
		LSD at 0.01 level = 7.11

[a] Averages for 5 replicates of 50 seed per treatment.
** Highly significant at 0.01 level.

treated alfalfa seed yielded a highly significant number of emerged plants as compared with nontreated seed. Arasan-treated seed (noninoculated) was significant in its yield of plants as compared with nontreatment. There were fifty seeds per treatment.

Table 5 consists of data showing the number of plants having nodules developed. In all cases the nodules were formed on lateral roots. In the calculation of percentage figures, the germination figures in table 4 were used, and employing transformation tables (Hayes and Immer), it was possible to carry out an analysis of variance for nodulation. The results are relatively conclusive. With a mean of 60.6 for Phygon-inoculated seed and a mean of 55.4 for Arasan-inoculated as compared with 37.4 for check-inoculated, it is evident that chemical treatment enhanced nodule formation in this test. Both the Phygon and Arasan figures for nodulation are highly significant over nontreated seed.

Table 5 also lists the results obtained with Chinese red cowpea seed treated with Phygon, Spergon, and Arasan (all at the 1.00 per cent dosage

rate), and the effects of these chemicals on nodulation. It is evident that nodulation was not inhibited by chemical treatments, even if treated seed was inoculated with "Nitragin" bacterial inoculum. Nodulation in this case was on lateral roots of plants resulting from chemically treated seed.

DISCUSSION OF RESULTS

Of the legumes tested, alfalfa, yellow hop clover, mungbean, and hairy vetch responded most consistently to seed treatment. On yellow hop clover, Spergon treatment was significantly superior to nontreatment and also to either Arasan or Dow 9B treatment in both field and greenhouse trials. Spergon, Phygon, Arasan, and Dow 9B were each significantly superior to nontreatment.

TABLE 5.—*Compatibility of seed treatment and inoculation with nodule bacteria, and the effect on nodulation, in alfalfa and cowpea*

Seed treatment	Inoculation with nodule bacteria	Mean number[a] of plants with nodules	
		Alfalfa	Cowpea
Spergon, 1 per cent	Inoculated	.....	63.6**
Spergon, 1 per cent	Not inoculated	.....	0.08
Phygon, 1 per cent	Inoculated	60.6**	71.2**
Phygon, 1 per cent	Not inoculated	0.0	0.0
Arasan, 1 per cent	Inoculated	55.4**	71.2**
Arasan, 1 per cent	Not inoculated	0.08	0.0
Control	Inoculated	37.4**	53.2**
Control	Not inoculated	0.06	4.5
	LSD at 0.05 =	7.91	26.19
	LSD at 0.01 =	10.70	34.04

[a] Averages for 5 replicates of 100 seeds of alfalfa and 100 seeds of Chinese red cowpea.

** Highly significant at 0.01 level.

The results obtained with the seed treatment of alfalfa were outstandingly consistent. Treatment with Phygon, which, prior to this work, had not been extensively tested on legumes, proved superior to nontreatment with high significance, and Arasan at the highest dosage also increased germination significantly. Hardening of seed coats, a characteristic that is associated with legume seed treated with heavy dosages of Phygon, did not affect the germination of alfalfa seed treated with 1.00 per cent dosages of Phygon. With three highly significant confirmatory tests conducted under nearly identical conditions, there is evidence of value in treating alfalfa seed with Phygon.

Mungbean, which is being grown on increasing acreages in Oklahoma, responded significantly to seed treatment with Phygon, Dow 9B, and Arasan, all of these treatments yielding highly significant results over nontreatment. Phygon, Arasan, and Dow 9B were also significantly superior to Spergon on mungbean seed. Phygon-treated seed yielded plants that

were strikingly taller and healthier in appearance than seed treated with Arasan, Dow 9B, or Spergon. It is also interesting to note the low germination figure of nontreated seed.

Hairy vetch seed treated with Arasan at all dosages, Phygon at 0.25 dosage, and Spergon at 0.25 dosage were significantly superior to nontreatment and to Dow 9B-treated seed. There was damage to seed treated with Dow 9B, appearing as disintegration and charring of the seed coats.

There was no indication in any of the tests with the legumes that chemical treatment of these seed inhibited nodule formation; there was, however, considerable evidence that nodulation is enhanced by Phygon, Arasan, Spergon, and Dow 9B with the majority of these seed. In the tests with alfalfa, Phygon- and Arasan-treated seed increased nodulation considerably, although it is interesting to find that the nodules were formed extensively on lateral roots, rather than on the tap root. This type of nodule formation was evident on the other legume seed treated with chemicals in all of the tests performed. On seed not treated with chemicals, nodules were formed on tap roots as well as on lateral roots.

SUMMARY

Alfalfa seed treated with Phygon at 1.00, 0.50, and 0.25 per cent dosages by weight of seed gave highly significant germination as compared with nontreated seed. Arasan at 1.00 per cent, Dow 9B at 1.00 per cent, and Spergon at 0.50 per cent dosage rates were all superior to nontreatment, but less effective than Phygon.

Mungbean seed treated with Phygon, Arasan, Dow 9B, and Spergon responded significantly to treatment, with Phygon at all dosages being most effective and yielding plants that were taller than seedlings from seed treated with other chemicals.

Chinese red cowpea seed treated with Arasan, Phygon, Spergon, or Dow 9B gave higher germination figures, significantly so, over nontreated seed.

Yellow hop clover seed responded most consistently to Spergon treatment as compared with Arasan, Phygon, Dow 9B, and Ceresan M treatments.

Hairy vetch seed showed highest germination where treated with Arasan, although Phygon-treated seed also was significantly superior, in germination, to nontreated seed.

Ceresan M proved to be injurious to all of the legume seed that were treated with this volatile chemical, and as a result was not included in experiments carried on in 1948.

Nodulation was not inhibited by chemical treatment of seed of alfalfa, mungbean, yellow hop clover, Chinese red cowpea, hairy vetch, and Austrian winter pea, when chemical treatment was followed by seed inoculation with "Nitragin" bacterial inoculum.

Nodulation of alfalfa and Chinese red cowpea was enhanced if seed of these legumes were treated with Phygon or Arasan and then inoculated

with "Nitragin." Chemically treated seed yielded more plants with nodules than nontreated seed.

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STILLWATER, OKLAHOMA

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DYING OF LIVE OAKS IN TEXAS[1]

A. A. Dunlap and A. L. Harrison

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The death of live oaks (*Quercus virginiana*) in Texas has been observed or reported in some twenty counties in the central part of the State, and has been called to the attention of plant pathologists over several years. The dying of 200 live-oak trees in the vicinity of Austin during 1933 and 1934 was described as the "live-oak disease" by Taubenhaus;[2] trees continued to die in that area in 1935. The nature of the causal agent has not been determined. Because the live oak is a valuable shade tree, the disease is of much economic importance in areas of its occurrence.

The environment in which the trees are growing appears to have little, if any, effect on the disease. Complaints of the dying of live oaks usually concern valuable trees near residences or trees that provide shade for livestock in farmyards (Fig. 1, A), although the disease is not restricted to these localities. It has been observed in forest land and even among trees near creeks where there is an abundance of moisture throughout the year. In surveys made in 1941 and 1942, covering several counties in south-central Texas, affected trees were found on both acid and highly alkaline soils, on sandy and heavy clay soils, on dry and wet soils, on hills and in valleys. Some of the trees were covered with Spanish moss and some were not. Young trees as well as older ones are affected. The disease occurs in somewhat restricted local areas and it seems to spread slowly to neighboring trees either in the same clump or in the same general locality.

Symptoms of the live-oak disease consist of yellowing or mottling of the leaves followed by death of certain branches or of the whole tree. Oftentimes the entire tree apparently becomes affected and it may die within a few weeks. If a tree is affected in only one branch at a time, one or two seasons may elapse before the entire tree is killed. In some instances a tree may appear healthy at the time of leaf shedding in early spring, but fail to put out new leaves at the usual time a few weeks later. Streaks of discolored wood have sometimes been seen in the roots of dying trees. Sprouts may appear from the roots of trees whose tops have died recently, but usually there is no persistent new growth by means of root or trunk sprouts.

Laboratory cultures of various parts of dying trees, including the discolored root tissues, have yielded no organism that has been proved or considered to be pathogenic; nor have a few grafting and budding experiments resulted in definite transmission of the symptoms. Studies of the dis-

[1] Published with the approval of the Director as Technical Article No. 1195, Texas Agricultural Experiment Station.

[2] Taubenhaus, J. J. Live-oak disease at Austin. Tex. Agr. Exp. Sta. 47th Ann. Rept., pp. 97–98. 1934. Also 48th Ann. Rept., pp. 99–100. 1935.

ease made thus far suggest that the trouble may be due to a virus, but this is purely conjecture.

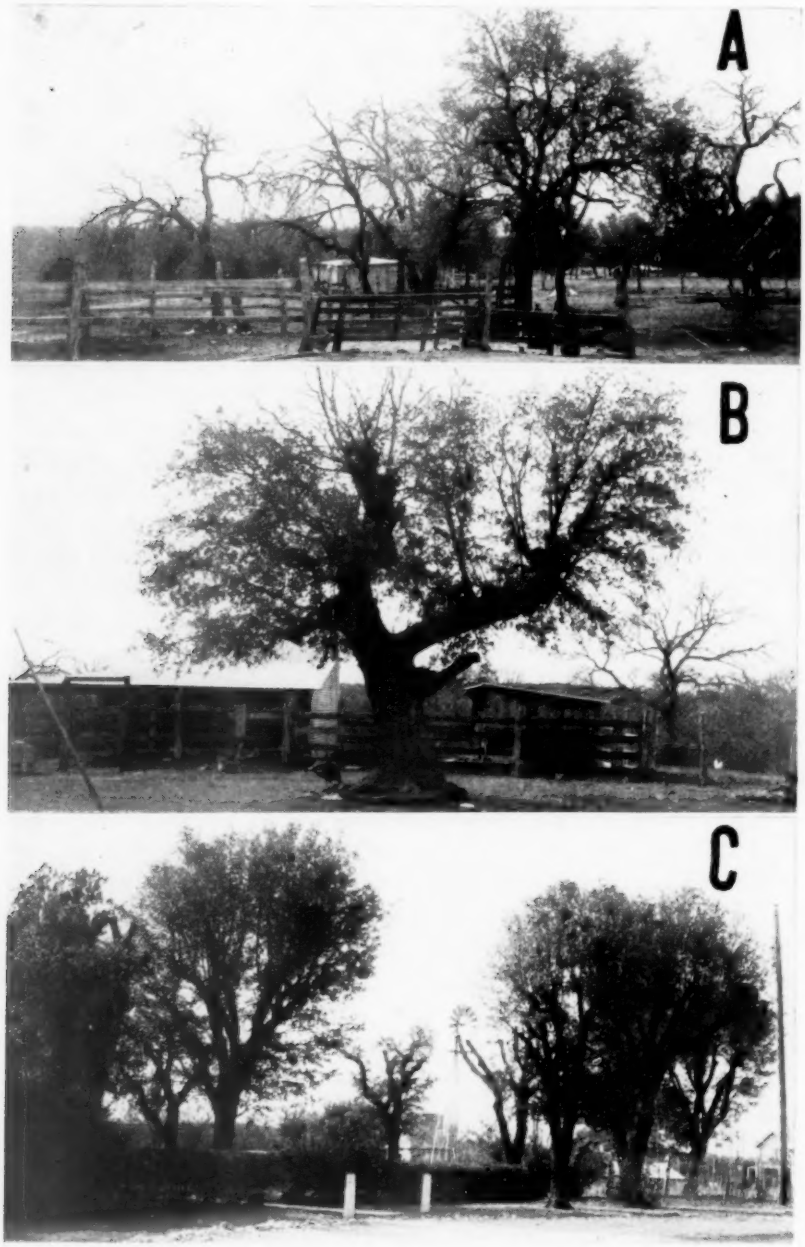

FIG. 1. Effects of pruning on death of live oaks in one farmyard. A. Only one tree remains alive in this group of seven live oaks which were not pruned. B. Large live oak that was topped when threatened with death in 1940. Dead nonpruned tree in background. C. New healthy tops formed on live oaks that were pruned when threatened with death in 1940. One trunk (center) is still alive although sprout growth was poor. One forked trunk (right center) failed to form sprouts after the pruning. Photographed April, 1948.

During the late fall of 1940, attention was called to a serious outbreak of the disease in Bosque County, near Clifton, Texas. At that time some experimental pruning was undertaken in an attempt to check the dying of live oaks around two ranch homes. The larger limbs were removed, in most cases, leaving only the main trunk of the tree standing (Fig. 1, B). The topped trees were observed from time to time, and, while some live oaks continued to succumb in the same general localities, only a very few trees died of the several that were severely pruned. Records taken in April, 1948, of the trees that were topped in November, 1940, showed that strong sprout growth from the treated trees had resulted in partial restoration of the top. Photographs of some of the trees, taken about 7½ years after severe pruning, are shown in figure 1, B and C. Among the 20 or more trees that were topped in this fashion only three were dead at the time of this last inspection. It is presumed that the disease was too far advanced in these three trees at the time of topping to permit recovery following the treatment (Fig. 1, C).

A resident of Yoakum, Texas, had lost four large live-oak trees near his residence previous to 1948. In the spring of that year, two more of the remaining live oaks also failed to produce new leaves following the usual defoliation in the late winter. One of these trees was pruned back to the main trunk late in April and the other was not pruned. During the summer new shoots appeared on the pruned trunk and on the stubs of the large branches. The nonpruned tree died completely without showing any growth of sprouts from the trunk.

The significance of the apparent recovery of the topped live-oak trees is not known but it is suggested that this treatment might prove effective against similar disorders in other woody plants.

SUMMARY

Live oaks have been observed to die more or less suddenly under a variety of environmental and seasonal conditions in central Texas. No causal agent has been associated with the so-called disease. Severe pruning of the main branches, at first appearance of symptoms, has apparently resulted in recovery and continued health of the trees in many instances.

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ROUGH BARK OF TUNG, A VIRUS DISEASE

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(Accepted for publication May 26, 1949)

In 1945 a tung tree, *Aleurites fordii*, having peculiar rough, black bark on the trunk and limbs and long, slender, twisted, willowy branches, was found in a tung orchard in St. Tammany Parish, Louisiana. An early symptom of this "rough bark" disease is a light bronze discoloration of the bark followed by the development of small widely spaced blisters on the surface. Later the bark turns dark in color and cracks into small irregularly shaped segments (Fig. 1, A). The diseased bark is in striking

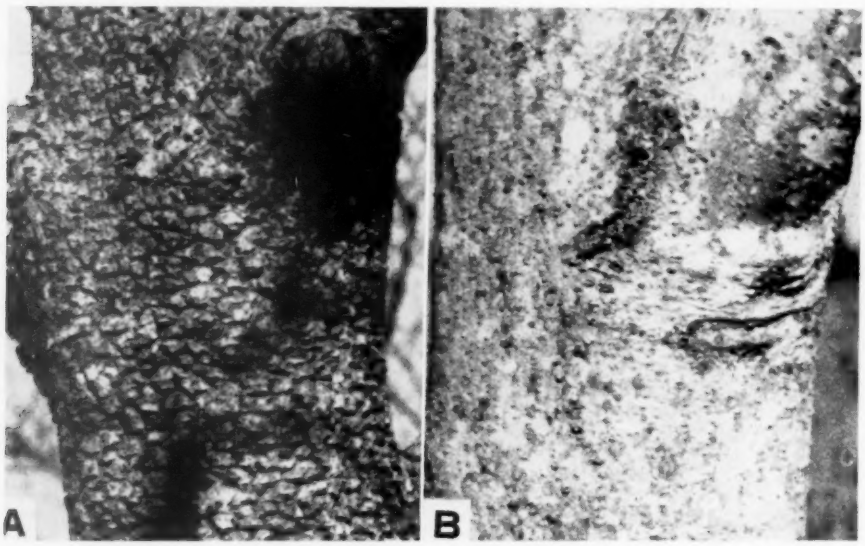

FIG. 1. A, tung tree affected with "rough bark" disorder. B, normal tung tree.

contrast to the smooth, light gray bark of a normal tung tree (Fig. 1, B). A second tung tree affected with rough bark disease was found in Pearl River County, Mississippi, in 1946.

Since the writer has found only two tung trees in the southern United States that are affected, the disease is not of present economic importance. Its effect upon the trees is difficult to judge, because they are seedlings and subject to much inherent variation. However, one of the trees is small and stunted, and both have long twisted internodes. Foliage is a normal dark green without variegation or mottling, but both trees appear to be subnormal in growth and production.

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The tissues and buds of affected plants have been examined for a possible insect infestation or causal organism, fungus or bacterium, but none has been found. It was therefore considered that the abnormality might be of genetic origin or caused by a virus.

To determine whether the disorder is caused by a virus, two budding experiments were started in 1946. In April, buds taken from the rough bark tree in St. Tammany Parish, Louisiana, were inserted by the T-bud method on 100 normal tung seedling rootstocks. On the same date, buds from normal trees were set in an equal number of normal tung rootstocks. The rootstocks were cut back to about 6 in. above the implanted buds in July, 1946. In September, 40 more rootstocks were budded with buds from the rough bark tree, and 20 with buds from normal tung trees. In May, 1947, the fall-budded rootstocks were cut back to about 6 in. above the implanted buds. The object was to force the implanted buds, but two or three suckers were permitted to grow from each rootstock to test the transmission of the disorder from the bud to the healthy rootstock. Observations on the 260 budded tung trees during 1947 and 1948 are summarized in table 1.

TABLE 1.—*Transmission of rough bark disease in tung trees budded in 1946*

Type of bud	Rootstock budded	Bud shoots as of November, 1948		Sucker shoots as of November, 1948[a]	
		Alive	With definite rough bark symptoms	With definite rough bark symptoms	With incipient rough bark symptoms
	Number	*Number*	*Number*	*Number*	*Number*
Rough bark	140	43	25	25[b]	18
Normal	120	40	0	0	0

[a] There were on the average 2 or 3 suckers per rootstock.
[b] Of the affected suckers, 14 arose above and 11 below the implanted bud.

The rough bark symptoms were slow to appear on the budded trees; the first definite symptoms were observed on the bud shoots in the second season, 1947. Four diseased suckers were also observed, two growing from above and two from below the implanted rough bark bud. In November of the third season, 1948, twenty-five of the bud shoots had definite rough bark symptoms. Fourteen sucker shoots that grew from above the implanted buds and eleven that grew from below developed rough bark symptoms. Eighteen more sucker shoots were observed developing the bronze bark appearance, characteristic of the early stages of the disease. No disease symptoms were observed on the trees budded with normal buds.

The transmission of the rough bark disease from the bud through the healthy rootstock to suckers developing above and below the implanted bud indicates that this disorder is a virus disease. To date no studies on possible insect vectors have been made.

SUMMARY

A disease that causes rough bark and willowy twisted branches of tung, *Aleurites fordii* Hemsl., is described. The disease, which has been named "rough bark," has been found in two widely separated orchards, one in Louisiana and one in Mississippi. Implanting buds from diseased shoots into healthy stocks has induced the disease in suckers developing from the rootstocks both above and below the implanted rough bark bud. In the absence of other causal organisms, the transmission of rough bark disease from the affected bud through the healthy rootstock to suckers indicates that this disorder is a virus disease.

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THE FUNGICIDAL ACTIVITY OF SUBSTITUTED PYRAZOLES AND RELATED COMPOUNDS

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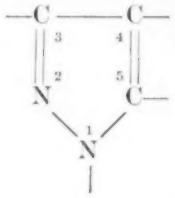
(Accepted for publication May 9, 1949)

Several types of nitrogenous compounds are fungitoxic. Among the more active are the quaternary ammonium compounds, 8-hydroxyquinoline derivatives, glyoxalidines, thiazoles containing salt-forming groups, and N-nitrosophthalimidine. All of them, except some of the quaternary ammonium compounds, contain the nitrogen in a heterocyclic ring.

In the course of a fundamental study on fungicides made in 1945, another group of heterocyclic nitrogen compounds was found to be active. These compounds, commonly known as pyrazoles, have the following nuclear structure:



Substitution of a nitroso group in the 4-position enhanced the activity of the pyrazoles, particularly of those compounds with an aryl group in the 1-position (27). Over 100 compounds were synthesized and tested in determining these relations. Data are presented on the performance of selected members in laboratory and greenhouse tests made to determine the effects of modification of the pyrazole nucleus, substitution in the 4-position, substitution in the 1-position, and substitution on a phenyl group in the 1-position on fungicidal and fungistatic activity. Data are also presented on the field performance of selected pyrazoles as seed, foliage, and fruit protectants.

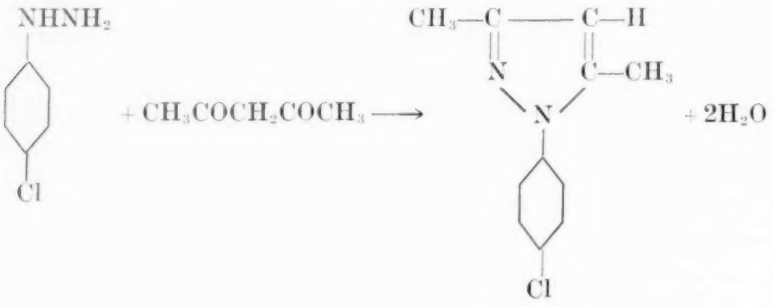
PREPARATION AND PROPERTIES OF COMPOUNDS TESTED

Only about one-third of the pyrazoles and related compounds used in defining the active groups are reported in this paper. These are representative and clearly demonstrate the relations found. Insofar as could be determined by the usual standards, these were, with only three exceptions, pure compounds. The names and outstanding characteristics of these compounds are listed in table 1.

Pyrazoles can be prepared by reacting hydrazine or a monosubstituted

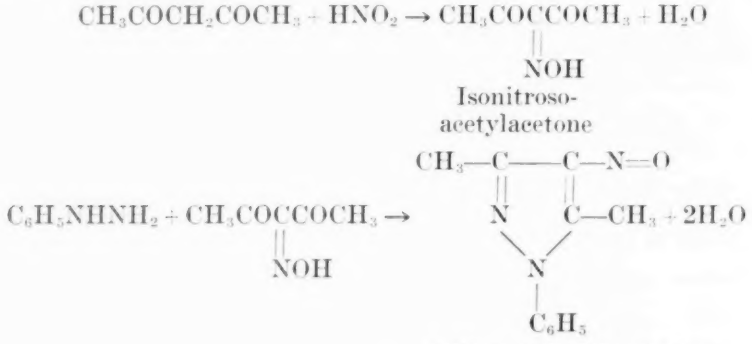
[1] The writers are deeply indebted to Ruth Schlessinger for aid in synthesizing many of the compounds that made this study possible and to John J. Natti, George E. O'Brien, Madeline V. Heaney, Adelaide Bornmann, and Mary Skipp for assistance in the field and laboratory evaluations.

hydrazine with a beta dicarbonyl compound. For example, the reaction of *p*-chlorophenylhydrazine and acetylacetone proceeds as follows:



1-*p*-Chlorophenyl-
3,5-dimethylpyrazole
(J54)

By choice of different monosubstituted hydrazines, the pyrazole molecule can be varied in the 1-position. By use of different beta dicarbonyl compounds, the substituents in the 3- and 5-positions can be modified. A nitroso group can be introduced into the 4-position by nitrosating the dicarbonyl compound before its reaction with the hydrazine:



1-Phenyl-3,5-dimethyl-
4-nitrosopyrazole
(J55)

Although three 4-nitrosopyrazoles were first prepared by this method in 1902 by Wolff *et al.* (31), one of these compounds probably was prepared by Knorr and Laubmann (14) as early as 1888 by direct nitrosation of 1,3,5-triphenylpyrazole.

The brief procedure of Wolff *et al.* (31) for the preparation of J55 was employed in preparing most of the 3,5-dimethyl-4-nitrosopyrazoles tested. An aqueous solution of isonitrosoacetylacetone was added to a stirred solution of an equimolar amount of the hydrazine in an equivalent quantity or more of hydrochloric acid diluted sufficiently to dissolve the hydrochloride formed. The reactions were allowed to proceed, generally under cooling, until the separation of the product was complete (2 to 24 hr.).

TABLE I.—*Properties of compounds investigated*

Compound Code	Compound Name	Appearance	Crystn. solvent[a]	M.p.[b] (or b.p.)	Analysis Calcd.	Analysis Found	Previous synthesis
				°C. *(uncor.)*	*Pct.*	*Pct.*	
30J	3,5-Dimethyl-4-nitrosopyrazole	dark blue ndls.	benzene	128			(31)
4K3	1,3,5-Trimethyl-4-nitrosopyrazole	blue ndls.	ligroin	80–81	N, 30.2	29.4	
39J	3-Methyl-4-nitroso-5-phenylpyrazole	dark green crystals	dil. alc.	149–150 (dec.)[c]			(25)
J55	1-Phenyl-3,5-dimethyl-4-nitroso-pyrazole	blue turquoise crystals	alcohol	95.5–96.5			(31)
0M6	1-*p*-Iodophenyl-3,5-dimethyl-4-nitrosopyrazole	green ndls.	alcohol	112	N, 12.8	12.6	
7K1	1-*p*-Bromophenyl-3,5-dimethyl-4-nitrosopyrazole	green ndls.	alcohol	123			(25)
J49	1-*p*-Chlorophenyl-3,5-dimethyl-4-nitrosopyrazole	green ndls.	alcohol	118	N, 17.8	17.7	
4L9	1-*p*-Fluorophenyl-3,5-dimethyl-4-nitrosopyrazole	fine blue-green ndls.	alcohol	116–117	N, 19.2	18.9	
35L	1-*m*-Chlorophenyl-3,5-dimethyl-4-nitrosopyrazole	light green crystals	alcohol	84	Cl, 15.1	15.3	
27L	1-*o*-Chlorophenyl-3,5-dimethyl-4-nitrosopyrazole	blue crystals	alcohol	117	Cl, 15.1	15.2	
3K4	1-(2,4-Dichlorophenyl)-3,5-dimethyl-4-nitrosopyrazole	green crystals	alcohol	89–90	N, 15.6	15.6	
3K3	1-(2,5-Dichlorophenyl)-3,5-dimethyl-4-nitrosopyrazole	green crystals	alcohol	99–100	N, 15.6	15.4	
4K5	1-*p*-Chlorophenyl-3(or 5)-methyl-4-nitroso-5(or 3)-*n*-hexylpyrazole	dark green oil			purity uncertain		
2K5	1-*p*-Tolyl-3,5-dimethyl-4-nitrosopyrazole	blue-green needles	alcohol	112			(25)
7K2	1-*p*-*tert*-Amylphenyl-3,5-dimethyl-4-nitrosopyrazole	green crystals	alcohol	92–93	N, 15.5	15.4	
9K5	1-*p*-Carboxyphenyl-3,5-dimethyl-4-nitrosopyrazole	light green crystals	alcohol	decompn. begins *ca.* 170[d]	N, 17.1	17.0	
8K2	1-*p*-Nitrophenyl-3,5-dimethyl-4-nitrosopyrazole	green ndls.	alcohol	131–132 (dec.)[e]	N, 22.8	22.2	
39L	1-*p*-Thiocyanophenyl-3,5-dimethyl-4-nitrosopyrazole	green ndls.	alcohol	105	N, 21.7	22.2	
36L	1-*p*-Sulfamylphenyl-3,5-dimethyl-4-nitrosopyrazole	green crystals	alcohol	decompn. begins *ca.* 189[f]	S, 11.4	11.7	
0M9	1-*p*-Xenyl-3,5-dimethyl-4-nitrosopyrazole	green crystals	alcohol	163 (dec.)[g]	N, 15.2	15.3	
19K	1-α-Naphthyl-3,5-dimethyl-4-nitrosopyrazole	pale blue crystals	alcohol	88–89	N, 16.7	16.6	
L63	1-Phenyl-3-methyl-4-nitroso-5-N-methylanilinopyrazole (not pure)	light green crystals					(19)

TABLE 1.—*(Cont.)*

Compound		Appearance	Crystn. solvent[a]	M.p.[b] (or b.p.)	Analysis		Previous synthesis
Code	Name				Calcd.	Found	
				°C. *(uncor.)*	*Pct.*	*Pct.*	
P90	Isonitrosoacetylacetone	white flat crystals	benzene-ligroin	76			(31)
16K	α-Naphthylhydrazine (not pure)	leaflets	water	113–115			(8)
5J0	*p*-Chlorophenylhydrazine hydrochloride	white crystals	abs. alc.	decomp. 225–226			(7)
E24	Phenylhydrazine[b]						
49K	4-Nitrosoantipyrine	dark green crystals	alcohol	decomp. suddenly 200			(12)
35J	1,3-Diphenyl-4,5-pyrazoledione-4-oxime	orange ndls.	dil. alc.	209 (dec.)[i]			(13)
J30	1-*p*-Chlorophenyl-3-methyl-4,5-pyrazoledione-4-oxime	yellow-orange hairlike ndls.	dil. alc.	180 (dec.)			(2)
J54	1-*p*-Chlorophenyl-3,5-dimethylpyrazole	liquid		(b_6 146–147)	Cl, 17.2	17.2	
L67	1-Phenyl-3,5-dimethyl-4-nitropyrazole	white crystals	dil. alc.	103			(31)
14K	1-Phenyl-3,5-dimethyl-4-amino-pyrazole monohydrate	white ndls.	water	66–67			(22)
53K	1-Phenyl-3,5-dimethyl-4-phenylazopyrazole	yellow ndls.	alcohol	62–63			(1)
1E1	3,5-Dimethylpyrazole[b]						
9K8	3-Methyl-5-ethoxypyrazole	white ndls.	ligroin	65–66			(30)
8K3	1-Phenyl-3-methyl-5-chloropyrazole	colorless liquid		(b_2 108–109)			(20)
9K7	1-Phenyl-3-methyl-5-ethoxypyrazole	white crystals	ligroin	37.5–38.5			(26)
13K	1-Phenyl-3-methyl-5-chloro-pyrazole methiodide	white ndls.	alcohol	236–237			(21)

[a] The 4-nitrosopyrazoles were dissolved in the solvent with minimum heating at 60° C. or less.

[b] Because of the instability of the 4-nitrosopyrazoles at elevated temperatures, the m.p.'s (or dec.p.'s) of some of them vary markedly with the temperature at which the capillary is introduced into the bath and the rate the bath is heated. The conditions employed for the determination of the recorded m.p.'s (or dec.p.'s) of these compounds are given.

[c] Detn. begun at 145° with a 3°/min. rise in temp.

[d] Detn. begun at 160° with a 4°/min. rise in temp.; beginning at *ca.* 170° the compound gradually changed from a green to a brown solid, the amt. of decompn. increasing with rise in temp.

[e] Detn. begun at 125° with a 3°/min. rise in temp.

[f] Detn. begun at 185° with a 3°/min. rise in temp.; beginning at *ca.* 189° the compound, with sintering, changed from green to brown, the amt. of decompn. increasing with rise in temp.

[g] Detn. begun at 160° with a 3°/min. rise in temp.

[h] Obtained from Eastman Kodak Co., "Eastman" grade.

[i] Varies with heating conditions; the recorded m.p. was observed when the detn. was begun at 180° with a 4°/min. rise in temperature. The literature reports m.p.'s varying from 199° to 214° for this compound.

Compound 30J was prepared by the procedure of Wolff *et al.* (31), 39J by the second procedure of Sachs and Alsleben (25), and 4K3 by adding a concentrated aqueous solution of isonitrosoacetylacetone to a cold aqueous solution of methylhydrazine sulfate neutralized with sodium carbonate. Compound 4K5 was prepared as follows: Enanthylacetone (prepared by treating a mixture of ethyl acetate and methyl *n*-hexyl ketone with sodium) was nitrosated in glacial acetic acid by addition of aqueous sodium nitrite. The resulting solution of isonitrosoenanthylacetone was added to an aqueous solution of *p*-chlorophenylhydrazine hydrochloride to give the oily product.

The methods for the preparation of the other compounds, if not the same as those given in the references to a previous synthesis in table 1, are as follows:

Compound J30, by adding aqueous sodium nitrite to a solution of 1-*p*-chlorophenyl-3-methyl-5-pyrazolone in 1:1 hydrochloric acid.

Compound J54, by adding *p*-chlorophenylhydrazine in portions to an equimolar quantity of acetylacetone with cooling. After the addition was completed, the mixture was heated in a boiling-water bath for 20 min.

Compound 14K, by the hydrogenation of J55 in alcohol at room temperature under an initial pressure of 52 lb. of hydrogen with Raney nickel as catalyst.

The solid compounds were purified by recrystallization from the solvents listed in table 1. The 4-nitrosopyrazoles were dissolved in the solvent with minimum heating at 60° C. or less. The moderate temperatures were adopted after J49 was observed to darken when dissolved in boiling alcohol and allowed to crystallize. The dark green product melted 2° lower than the pure compound. Likewise 30J, a dark blue compound when pure, was dark greenish-blue when crystallized from boiling benzene.

Further evidence that the 4-nitrosopyrazoles are unstable at elevated temperatures was obtained when their melting points were determined. Two of them decomposed before showing evidence of melting. Others decomposed while melting. On this account, the rate of heating had to be controlled to obtain reproducible results (Table 1, footnote b).

The stability of 2K5 (m.p. 112°) at 71° C. was determined. There was no change in melting point in 10 days; after 12 days the melting point was 110–111°; after 17 days, 108–111°; after 20 days, 90–95°. The color changed from blue-green to brown during the 20-day period.

The 4-nitrosopyrazoles react readily with dilute sodium hydroxide. Sachs and Alsleben (25) noted that a small amount of sodium hydroxide solution added to a boiling alcohol solution of 1,5-diphenyl-3-methyl-4-nitrosopyrazole changed the solution from green to red-brown. The product was the azoxy compound.

Similar color changes have been observed when aqueous sodium hydroxide was added to an aqueous solution of 4K3 or an alcohol solution of 2K5 at room temperature. The solutions changed slowly from blue and

g Detn. begun at 160° with a 3°/min. rise in temp.
h Obtained from Eastman Kodak Co., "Eastman" grade.
i Varies with heating conditions; the recorded m.p. was observed when the detn. was begun at 180° with a 4°/min. rise in temperature. The literature reports m.p.'s varying from 199° to 214° for this compound.

blue-green, respectively, to brown and could not be restored to the original color by acidification.

EXPERIMENTAL PROCEDURES

A series of standard bioassays was made on all chemicals. The chemicals were suspended or dissolved in distilled water at a concentration of 2000 ppm by using Emulphor EL, a commercial surface-active agent, where necessary and thoroughly agitating in a Waring Blendor. Part of the stock suspension was diluted to 1000 ppm and used for spore germination tests on glass slides by the test tube dilution method recommended by the Committee of The American Phytopathological Society (3). The concentrations specified in the tables refer to initial concentration before diluting 4 volumes with 1 volume of orange juice filtrate and spore suspension. Spores from 7-day-old cultures of *Alternaria oleracea* Milb. (17) and *Sclerotinia americana* (Worm.) Nort. and Ezek. were used for test purposes. Germination records were taken after 18 hours of incubation at 72° F. Sufficient extra spores were counted to provide the equivalent of 100 per cent germination in the untreated controls.

Another aliquot of the chemical suspension was diluted serially in distilled water to provide a dosage series for testing ability to protect tomato foliage against infection by the early blight fungus, *Alternaria solani* (E. and M.) V. and G. A modification of the method described by McCallan and Wellman (18), employing two or three potted 6-in. plants of the variety Bonny Best for each concentration of chemical, was used. The plants were sprayed for 20 sec. at 20 lb. air pressure while being rotated in a spray chamber on a turntable 56 in. from the nozzle of the spray gun. After the spray deposit was thoroughly dry (3 to 16 hr.), the plants and comparable unprotected controls were sprayed with a suspension containing about 20,000 spores of *A. solani* per ml. The DeVilbiss atomizer used discharged 11 ml. during a 20-sec. period of exposure. The plants were held for 19 to 23 hr. at 75° F. and 99 per cent relative humidity to permit spore germination and infection before returning them to the greenhouse. Records were taken 3 to 6 days later on the number of lesions produced on the 15 major leaflets of the three youngest fully expanded leaves. The data were converted to percentage of control on the basis of the average number of lesions on four unprotected plants and plotted on logarithmic-probability paper to obtain an LD95 value. With very few exceptions, a satisfactory linear dosage-response curve was obtained by this procedure.

Seed protectant tests were made in composted soil in the greenhouse and on a well-drained silt loam field, both of which were known to be severely infested with seed-decay and damping-off fungi (*Pythium* spp. and *Rhizoctonia* spp.). Each chemical was applied in dust form at three dosage levels to seed of peas, corn, beets, and spinach. The chemical and seed, each weighed to the nearest 0.01 gm., were placed in a jar, rotated for 30 min.

at 28 r.p.m. on a vertical turntable, and reweighed to determine the actual dosage of chemical adhering to the seed. In greenhouse trials, lots of 25 seeds of corn and peas or 50 seeds of spinach and beets were sown at random in each of eight replications. The seed was covered to a uniform depth, water was added, and the flats held in a chamber at 55° F. and 99 per cent humidity until the seedlings emerged. They were then removed to a greenhouse at 65° to 80° F. where they were held until emergence records were taken about ten days later. Field trials were made with five replications of 100 seeds each for corn, peas, and spinach, and eight replications for beets.

Field spray treatments were applied by conventional power sprayers capable of developing 350 lb. pressure. Apples were sprayed with an orchard gun from the ground at the rate of 10 to 25 gal. per 20-year-old tree, depending upon the size. Tomatoes were sprayed at the rate of about 200 gal. per acre with a portable boom equipped with five nozzles per row. Potatoes were sprayed in the same fashion with a four-nozzle boom. Potatoes of the variety Green Mountain were grown in 20-ft. parallel rows and six single-row replicates were used. Tomatoes of the variety John Baer were grown in a near-by plot, with seven plants per 21-ft. row in a planting spaced 5 by 3 ft. Five single-row replicates of each treatment were used.

In order to have a standard of comparison in all tests, either tetrachloro-*p*-benzoquinone (Spergon) or 2,3-dichloro-1,4-naphthoquinone (Phygon) was included. These quinones are known to be highly active fungicides capable of satisfactory field performance on seeds and foliage; so anything equivalent or superior to them is considered to have definite practical possibilities.

EXPERIMENTAL RESULTS

Effect of Substitution in the 4-Position

In preliminary tests pyrazoles with a nitroso group substituted in the 4-position were found to be much more active than comparable 4-unsubstituted compounds. A number of compounds were synthesized with nitroso, nitro, amino, phenylazo, or other groups in this position. The ability of six of these compounds to inhibit germination of Alternaria and Sclerotinia spores on glass slides was determined. The results may be summarized briefly as follows. Compound J54 (without substitution in the 4-position and with a *p*-chlorophenyl group in the 1-position) completely inhibited germination of both types of spores at 1,000 ppm, had no effect on Alternaria spores at lower concentrations, permitted 85 to 89 per cent germination of Sclerotinia at 100 and 10 ppm, and had no effect on Sclerotinia at a dosage of 1 ppm. When the analogous compound (J49) with a nitroso group substituted in the 4-position was tested, both types of spores were completely inhibited at dosages as low as 1 ppm. There was 90 and 99 per cent germination at 0.1 ppm but no effect at lower concentrations.

This observation on the effectiveness of pyrazoles with a 4-nitroso group was further investigated by synthesizing a series of compounds with either

a nitroso (J55), amino (14K), phenylazo (53K), or nitro (L67) group in the 4-position of 1-phenyl-3,5 dimethylpyrazole. The compound with the nitroso group was about as effective as J49 and decisively more effective than any of the others. Compound 53K failed to inhibit spores of either fungus at concentrations of 1000, 100, 10, or 1 ppm. Compound 14K inhibited germination at 1000 ppm but failed at 100, 10, and 1 ppm. Compound L67 with a 4-nitro group was only slightly more active than 14K. These data suggest that the nitroso group has some unusual ability not common to other nitrogen groups when substituted in the 4-position on the pyrazole nucleus, and it is effective irrespective of the type of substituent in the 1-position. The nitroso group accentuates fungitoxicity about a thousandfold.

Substitution of other groups in the 3- and 5-positions had little or no effect. For example, 3-methyl-5-ethoxypyrazole (9K8) inhibited very few spores at 1000 ppm, and 1-phenyl-3-methyl-5-ethoxypyrazole (9K7) did not inhibit germination at concentrations below 10 ppm. 1-Phenyl-3-methyl-5-chloropyrazole (8K3) and its methiodide (13K) were relatively ineffective. The full value of such compounds can be realized only by substitution of a nitroso group in the 4-position. 1-Phenyl-3-methyl-4-nitroso-5-N-methylanilinopyrazole (L63) completely inhibited spore germination at 1 ppm.

Representatives of the above compounds were tested on tomato foliage as protectants against *Alternaria solani*. The weaker compounds were tested over a dosage range of 2000 to 128 ppm and the more active over a range of 256 to 2 ppm. The data on the percentage of disease control are summarized in table 2. For some reason, probably due to the physiological

TABLE 2.—*Effect of varying the substituent in the 4-position of 3,5-dimethylpyrazoles on ability to prevent infection of tomato foliage by Alternaria solani*

Code	Substituent in		Blight control[a] at concentration (ppm)								
	4-position	1-position	2000	1024	512	256	128	64	32	16	8
			Pct.	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*
J54	Hydrogen	*p*-Chlorophenyl	.....	45.6	38.0	.....	38.1	.....	.....	.....	.....
J49	Nitroso	*p*-Chlorophenyl	.....	.....	.....	.....	100.0	99.9	99.6	93.0	90.4
J55	Nitroso	Phenyl	.....	.....	.....	99.6	99.8	99.3	96.3	89.8	73.2
14K	Amino	Phenyl	92.1	88.1	67.4	.....	36.7	.....	.....	.....	.....
53K	Phenylazo	Phenyl	85.8	73.2	59.9	.....	43.8	.....	.....	.....	.....
2,3-Dichloro-1,4-naphthoquinone			.....	.....	.....	99.9	99.6	98.6	92.0	59.9	56.7

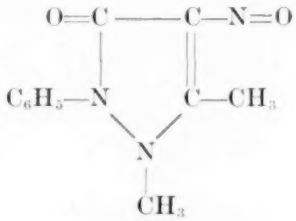
[a] Unsprayed plants had an average of 420 lesions.

vigor of the plants, the disease control was definitely superior to that observed in preliminary trials on these same compounds. The relative position of the various materials, however, remained constant; so the data are considered reliable. When the data were plotted on logarithmic-probability paper, very good straight-line dosage-response curves were obtained. The

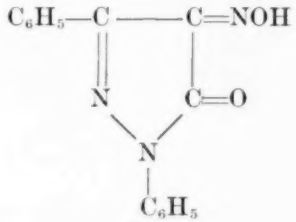
estimated LD95 values were: J49, 10.4 ppm; J55, 25.5 ppm; 14K, 2300 ± ppm; and 53K, 5000 ± ppm. The higher LD95 values are only rough approximations, since they were obtained by extrapolation so far beyond the available data. Compound J54 caused considerable foliage necrosis at the two higher dosages, and its LD95 value, if any, was something over 10,000 ppm. These data, in general, agree with the observation on spore inhibition recorded above, except that the superiority of J49 over J55 would not have been anticipated from the slide data. All of the materials, except J54, apparently were noninjurious to foliage in this and various other tests made over a two-year period.

Modification of the Pyrazole Nucleus

In view of the high fungitoxic activity of the 4-nitrosopyrazoles, studies were undertaken to determine the effectiveness of closely related compounds such as 49K and 35J:



49K



35J

Compound 49K differs from the 4-nitrosopyrazoles in that the 4-nitroso group is attached to a 3-pyrazolone; 35J differs in that the nitroso group has been replaced by an isonitroso group, which is attached to a 5-pyrazolone.

These compounds and J30 were compared to the 4-nitrosopyrazoles J49 and J55 and several related compounds on glass slides and tomato foliage. The data summarized in tables 3 and 4 show J30 and 35J were good fungicides (28) but less effective than the 4-nitrosopyrazoles. Compound 49K was only weakly fungicidal on foliage and had little effect on spores in the glass slide tests.

The tomato foliage data show approximate LD95 values for 49K of 10,000 ± ppm; 35J, 4500 ± ppm; J49, 31.5 ppm; and J55, 60 ppm. In view of these differences, further study was directed toward improving the 4-nitrosopyrazoles by varying the substituents in the 1- and 5-positions rather than toward altering the essential structure of the nucleus.

Effect of Substitution in the 1-Position

The possibility of improving the fungicidal activity of the 4-nitrosopyrazoles by altering the substituent in the 1-position was next investigated. Compounds were synthesized with methyl (4K3), phenyl (J55), and α-naphthyl (19K) groups in the 1-position of 3,5-dimethyl-4-nitrosopyrazole (30J).

TABLE 3.—*Relative effectiveness of several pyrazoles and pyrazolones and chemicals used in synthesizing pyrazoles in inhibiting germination of fungus spores on glass slides*

Compound tested		Fungus tested	Spore germination[a] at conc. (ppm)					
Code	Name		1000	100	10	1	0.1	0.01
			Pct.	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*
49K	4-Nitrosoantipyrine	Alter.	99	100	100	100	.....	.....
		Scler.	100	100	100	100	.....	.....
35J	1,3-Diphenyl-4,5-pyrazoledione-4-oxime	Alter.	0	0	100	100	.....	.....
		Scler.	0	100	100	100	.....	.....
J30	1-*p*-Chlorophenyl-3-methyl-4,5-pyrazoledione-4-oxime	Alter.	0	48	97	100	.....	.....
		Scler.	0	62	100	100	.....	.....
J55	1-Phenyl-3,5-dimethyl-4-nitrosopyrazole	Alter.	.....	.....	0	0	99	100
		Scler.	.....	.....	0	0	95	100
J49	1-*p*-Chlorophenyl-3,5-dimethyl-4-nitrosopyrazole	Alter.	.....	.....	0	0	100	100
		Scler.	.....	.....	0	0	100	100
5J0	*p*-Chlorophenylhydrazine hydrochloride	Alter.	.....	.....	100	100	100	100
		Scler.	.....	.....	77	98	100	100
19K	1-α-Naphthyl-3,5-dimethyl-4-nitrosopyrazole	Alter.	.....	.....	0	0	97	100
		Scler.	.....	.....	0	0	100	100
16K	α-Naphthylhydrazine	Alter.	.....	98	100	100	.....	.....
		Scler.	.....	89	100	100	.....	.....
E24	Phenylhydrazine	Alter.	0	0	73	100	.....	.....
		Scler.	0	0	0	5	.....	.....
P90	Isonitrosoacetylacetone	Alter.	98	100	100	100	.....	.....
		Scler.	100	100	100	100	.....	.....
2,3-Dichloro-1,4-naphthoquinone		Alter.	.....	.....	0	0	98	100
		Scler.	.....	.....	0	0	43	100

[a] Germination of controls: Alternaria, 98; Sclerotinia, 93.

TABLE 4.—*Relative effectiveness of several pyrazoles and pyrazolones and chemicals used in synthesizing pyrazoles in preventing infection of tomato foliage by Alternaria solani*

Compound tested[a]	Blight control[b] at concentration (ppm)										
	2000	1024	512	256	128	64	32	16	8	4	2
	Pct.	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*
49K	65.5	25.5	25.1	18.2	18.2	12.3	.....	.....	.....	.....	.....
35J	90.2	75.0	77.9	66.5	53.2	48.1	.....	.....	.....	.....	.....
J55	.....	.....	.....	99.1	99.1	94.6	87.0	71.8	47.6	.....	.....
J49	.....	.....	.....	.....	99.8	98.6	96.6	85.0	58.4	37.1	30.0
5J0	88.5	85.1	51.3	56.0	42.7	14.3	.....	.....	.....	.....	.....
19K	.....	.....	.....	100.0	99.5	97.1	97.8	94.4	79.7	57.4	45.1
E24	41.5	41.5	46.6	12.4	5.3	18.9	.....	.....	.....	.....	.....
P90	22.9	19.2	16.8	19.4	27.1	4.8	.....	.....	.....	.....	.....
Phygon	.....	.....	.....	99.7	99.3	97.6	90.9	65.5	28.4	.....	.....

[a] See table 3 for chemical name.
[b] Unsprayed plants had an average of 296 lesions.

Data on these compounds and two others with different substituents in the 5-position are summarized in tables 5 and 6.

TABLE 5.—*Effect of varying the substituents in the 1- and 5-positions of 3-methyl-4-nitrosopyrazole on ability to inhibit germination of fungus spores on glass slides*

Code	Substituent in		Fungus tested	Spore germination[a] at conc. (ppm)			
	1-position	5-position		10	1	0.1	0.01
				Pct.	*Pct.*	*Pct.*	*Pct.*
30J	Hydrogen	Methyl	Alter.	100	100	100	100
30J	Hydrogen	Methyl	Scler.	100	100	100	100
4K3	Methyl	Methyl	Alter.	0	21	100	100
4K3	Methyl	Methyl	Scler.	0	39	100	100
J55	Phenyl	Methyl	Alter.	0	0	15	100
J55	Phenyl	Methyl	Scler.	0	0	97	100
19K	α-Naphthyl	Methyl	Alter.	0	0	100	100
19K	α-Naphthyl	Methyl	Scler.	0	0	98	100
39J	Hydrogen	Phenyl	Alter.	100	100	100	100
39J	Hydrogen	Phenyl	Scler.	10	98	100	100
4K5	*p*-Chlorophenyl	*n*-Hexyl[b]	Alter.	98	100	100	100
4K5	*p*-Chlorophenyl	*n*-Hexyl[b]	Scler.	99	100	100	100
Dichloronaphthoquinone			Alter.	0	0	100	100
			Scler.	0	0	54	100

[a] Germination of controls: Alternaria, 96 per cent; Sclerotinia, 91 per cent.
[b] Possibly in 3-position with methyl group at 5.

TABLE 6.—*Effect of varying the substituents in the 1- and 5-positions of 3-methyl-4-nitrosopyrazole on ability to prevent infection of tomato foliage by Alternaria solani*

Code	Substituent in		Blight control[a] at conc. (ppm)								
	1-position	5-position	2000	1024	512	256	128	64	32	16	8
			Pct.	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*
30J	Hydrogen	Methyl	91.6	91.9	87.9	.....	59.7	.....	.....	.....	.....
J55	Phenyl	Methyl	.....	.....	.....	99.6	99.8	99.3	96.3	89.8	73.2
19K	α-Naphthyl	Methyl	.....	.....	.....	.....	99.6	100.0	99.3	98.5	92.7
39J	Hydrogen	Phenyl	93.3	85.6	81.8	.....	77.3	.....	.....	.....	.....
4K5	*p*-Chlorophenyl	*n*-Hexyl[b]	39.9	78.5	4.0	.....	55.5	.....	.....	.....	.....
Dichloronaphthoquinone			.....	.....	.....	99.9	99.6	98.6	92.0	59.9	56.7

[a] Unsprayed plants had an average of 420 lesions.
[b] Possibly in 3-position with methyl group at 5.

The records of spore germination show that 30J was ineffective at the low dosages used in this test, while the substitution of a phenyl or α-naphthyl group increased activity at least a hundredfold. The methyl group was not so effective. The substitution of a phenyl group for the methyl group in the 5-position of 30J (see 39J) had very little effect. The phenyl group is most effective when attached to the nitrogen in the 1-position.

The data obtained on tomato foliage substantiate the observations on spore germination on glass slides. When the data on blight control were plotted on logarithmic-probability paper, a satisfactory straight-line dosage

response was obtained. The estimated LD95 values were: 30J, 2500 ± ppm; J55, 25.5 ppm; 19K, 10.4 ppm; 39J, 2500 ± ppm; and 4K5, > 10,000 ppm. There was not sufficient 4K3 available for a foliage test in this series; but in a comparable preceding test it had an LD95 value of 10,000 ppm or slightly less. Hence, it may be considered a weak fungicide.

These studies demonstrate that an aryl group must be substituted in the 1-position if a 4-nitrosopyrazole is to attain maximum effectiveness. Of the two compounds containing aryl groups investigated, the α-naphthyl derivative was more effective. It is, however, more difficult to synthesize than the phenyl derivative; so further studies on modification of the aryl substituent were restricted to the phenyl group.

Effect of Substitution on the 1-Phenyl Group

Since the foregoing studies had shown that 4-nitroso and 1-aryl groups were requisites for maximum fungicidal activity, the next series of studies was devoted to determining the effect of substitution on the 1-phenyl group. The various compounds listed in table 7 were synthesized and tested for ability to inhibit spore germination on glass slides.

The first five compounds listed were chosen in order to determine the effect of the halogens substituted in the *para* position on fungicidal activity. The data on glass slides show that the unsubstituted compound (J55) was the most effective, since spore germination was inhibited by a concentration as low as one part in more than a billion of water. These data are considered somewhat misleading, since in several other tests J55 was approximately equivalent to the *p*-chloro derivative (J49). The material (J55) is somewhat water-soluble and it is believed that small differences in manipulation of the test affected the results. The *p*-chloro derivative was about as effective as the *p*-fluoro derivative, but was approximately ten times more potent than the corresponding bromo and iodo compounds. This observation confirms data obtained in several preceding tests but not reported here.

The shifting of the chlorine to other positions on the phenyl ring materially altered the fungicidal effectiveness. In comparison to the *p*-chloro derivative (J49), the *m*-chloro derivative (35L) was only slightly less effective, while the *o*-chloro compound (27L) was appreciably less active. The two dichloro derivatives (3K3 and 3K4) were only about one-tenth as active as J49, the *p*-chloro derivative.

These effects of halogen substitutions are of particular interest in view of the important role played by chlorine in the many new organic fungicides and insecticides. The chlorine probably contributes to the blocking of essential functions by *p*-dichlorobenzene, Chlordane, DDT, metallic trichlorophenolates, chlorinated quinones, *etc.*

In order to determine the effectiveness of other substituents on the 1-phenyl group, phenylhydrazines with methyl, *tert*-amyl, nitro, thiocyano, carboxyl, sulfamyl, and phenyl groups in the *para* position were reacted

TABLE 7.—*Effect of various substituents on the phenyl group in 1-phenyl-3,5-dimethyl-4-nitrosopyrazole on ability to inhibit germination of fungus spores on glass slides*

Compound tested		Test fungus	Germination[a] at concentration (ppm)						
Code	Substituent		100	10	1	0.1	0.01	0.001	0.0001
			Pct.	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*
J55	None	Alter.	0	0	0	0	0	0	94
J55	None	Scler.	0	0	0	0	32	10	96
0M6	*p*-Iodo	Alter.	0	0	0	99	98	100	100
0M6	*p*-Iodo	Scler.	0	0	9	82	91	95	100
7K1	*p*-Bromo	Alter.	0	0	0	2	100	100	100
7K1	*p*-Bromo	Scler.	0	0	0	94	100	100	100
4L9	*p*-Fluoro	Alter.	0	0	0	0	95	100	100
4L9	*p*-Fluoro	Scler.	0	0	0	1	97	100	100
J49	*p*-Chloro	Alter.	0	0	0	0	99	97	100
J49	*p*-Chloro	Scler.	0	0	0	0	90	100	100
35L	*m*-Chloro	Alter.	0	0	0	0	91	100	100
35L	*m*-Chloro	Scler.	0	0	0	5	67	88	100
27L	*o*-Chloro	Alter.	0	0	0	0	96	100	100
27L	*o*-Chloro	Scler.	0	0	0	60	94	100	100
3K4	2,4-Dichloro	Alter.	0	0	0	100	100	100	100
3K4	2,4-Dichloro	Scler.	0	0	0	100	100	100	100
3K3	2,5-Dichloro	Alter.	0	0	0	97	100	100	100
3K3	2,5-Dichloro	Scler.	0	0	0	96	100	100	100
2K5	*p*-Methyl	Alter.	0	0	0	0	88	100	100
2K5	*p*-Methyl	Scler.	0	0	0	0	88	100	100
7K2	*p*-*tert*-Amyl	Alter.	0	0	0	100	100	100	100
7K2	*p*-*tert*-Amyl	Scler.	0	0	0	86	100	100	100
8K2	*p*-Nitro	Alter.	0	0	0	81	99	100	100
8K2	*p*-Nitro	Scler.	0	0	0	93	97	100	100
39L	*p*-Thiocyano	Alter.	0	0	3	100	100	100	100
39L	*p*-Thiocyano	Scler.	0	0	0	96	100	100	100
9K5	*p*-Carboxy	Alter.	0	96	99	97	100	100	100
9K5	*p*-Carboxy	Scler.	0	93	94	100	100	100	100
36L	*p*-Sulfamyl	Alter.	100	100	100	100	100	100	100
36L	*p*-Sulfamyl	Scler.	90	100	100	100	100	100	100
0M9	*p*-Phenyl	Alter.	0	36	86	100	100	100	100
0M9	*p*-Phenyl	Scler.	69	95	95	90	96	100	100
Tetrachlorobenzoquinone		Alter.	0	0	66	98	100	100	100
		Scler.	0	0	63	100	100	100	100

[a] Germination of controls: Alternaria, 96 per cent; Sclerotinia, 92 per cent.

with isonitrosoacetylacetone. The series of 3,5-dimethyl-4-nitrosopyrazoles obtained was tested. Some of these, such as the phenyl (0M9), sulfamyl (36L), and carboxy (9K5) derivatives, were mediocre. The one interesting discovery in this series was that the *p*-methyl derivative (2K5), *i.e.*, the compound with a *p*-tolyl radical in the 1-position of the pyrazole ring, was fully as effective as J49 if not superior to it. Apparently the number of carbon atoms in the *para* position cannot be increased very much if this

extreme activity is to be maintained, since the compound with a *tert*-amyl group (7K2) was less effective.

The full significance of these data can be realized by comparing the performance of these new fungicides to that of tetrachlorobenzoquinone. Only the three least active members were inferior to the standard. The better 4-nitrosopyrazoles were almost 100 times as potent. Spergon has a rating somewhat lower than 2,3-dichloro-1,4-naphthoquinone in glass slide tests, but is equivalent or superior to many other successful fungicides.

Duplicate tests were made on tomato foliage using one series of plants inoculated immediately after spraying and the other, 36 hr. later. Detailed data on the first test and the estimated LD95 values from both tests are presented in table 8. The LD95 values in the second test were consistently

TABLE 8.—*Effect of various substituents on the phenyl group in 1-phenyl-3,5-dimethyl-4-nitrosopyrazole on ability to prevent infection of tomato foliage by Alternaria solani*

Compound tested		Blight control[a] at concentration (ppm) in Test 1									LD95 value	
Code	Substituent	960	480	240	120	60	30	15	7.5	3.8	Test 1	Test 2
		Pct.	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*	ppm	ppm
J55	None	.....	.....	99.8	99.3	94.4	78.2	74.0	.....	.....	62	120
0M6	*p*-Iodo	.....	.....	88.4	91.3	77.0	37.6	23.1	.....	.....	170	250
7K1	*p*-Bromo	.....	.....	.....	81.6	77.5	39.6	17.8	15.9	.....	400	230
4L9	*p*-Fluoro	.....	.....	.....	100.0	98.8	92.5	68.3	18.1	.....	37	100
J49	*p*-Chloro	.....	.....	.....	.....	97.6	95.2	92.0	42.0	37.0	28	35
35L	*m*-Chloro	.....	.....	.....	.....	97.8	93.5	89.1	37.4	14.7	34	72
27L	*o*-Chloro	.....	.....	.....	97.1	96.4	92.7	77.3	72.7	.....	60	100
3K4	2,4-Dichloro	.....	.....	99.8	97.6	85.0	43.0	34.5	.....	.....	92	120
3K3	2,5-Dichloro	.....	.....	98.5	97.3	88.4	51.2	47.0	.....	.....	100	120
2K5	*p*-Methyl	.....	.....	.....	.....	97.8	89.1	93.2	65.2	25.0	30	56
7K2	*p-tert*-Amyl	.....	.....	.....	.....	86.9	65.4	32.3	12.2	4.3	100	150
8K2	*p*-Nitro	.....	93.2	87.7	72.0	47.1	50.0	.....	.....	.....	560	900
39L	*p*-Thiocyano	.....	.....	79.9	82.4	83.6	31.4	5.7	.....	.....	220	750±
9K5	*p*-Carboxy	91.1	55.3	49.5	55.3	72.4	.....	.....	.....	.....	1500	900±
36L	*p*-Sulfamyl	.....	91.8	98.3	79.2	65.7	37.2	.....	.....	.....	560	820
0M9	*p*-Phenyl	75.1	72.4	68.8	10.5	.....	.....	.....	.....	.....	∞	3500±
Dichloronaphthoquinone		.....	.....	100.0	99.3	78.5	25.3	8.9	.....	.....	82	115

[a] Unsprayed plants had an average of 414 lesions.

higher than in the first one, but the relative values were consistent. The compounds fell in about the same relative positions as they did on glass slides. Compounds 2K5 and J49 were superior, with LD95 values of about 30 ppm. The other halogen-containing derivatives had LD95 values of 34 to 400 ppm. Compounds 0M9, 36L, 9K5, 39L, and 8K2 were definitely inferior to the others.

These data on foliage are considered reliable. They are typical of the excellent disease control obtained without visible foliage injury over a testing period of 18 months. In these preceding tests 2K5 had LD95 values of 25 ppm on two occasions, 30 ppm in three tests, and 35 ppm twice. Compound J49 had values of 20 ppm once, 25 ppm six times, 30 ppm four

times, 35 ppm once, and 55 ppm once. These values obtained on plants of different age and vigor agree well with the range of 28 to 56 ppm reported in table 8. Compound J55 did not rate so consistently effective in six preceding tests, since its LD95 values were 55, 80, 140, 145, 150, and 170 ppm. The other compounds listed in table 8 gave disease control in preliminary trials comparable to that reported.

Attention is particularly directed to the performance of 1-*p*-sulfamylphenyl-3,5-dimethyl-4-nitrosopyrazole (36L). The material had negligible ability to inhibit spore germination on glass slides. It was somewhat more effective as a foliage protectant in spite of its poor comparative position in relation to the other compounds. There are several fungicides used commercially that would do no better in this particular test. These data, however, offer no suggestion that this might be an exceptionally effective fungicide on seeds, as will be shown in table 11.

These studies on the substituents on the 1-phenyl group climaxed the efforts to create a superior fungicide of the 4-nitrosopyrazole class. The success of this effort may be realized by making comparisons with the two quinone fungicides used as standards. Thirteen of the 16 compounds reported in table 7 were more toxic to spores on glass slides than was tetrachlorobenzoquinone (Spergon), a widely used commercial product. In the foliage tests there were seven compounds that were fully as effective as 2,3-dichloro-1,4-naphthoquinone (Phygon), an outstanding fungicide that is finding many practical applications (4, 5, 6, 9, 23).

The outstanding representatives of this class are 1-*p*-tolyl-3,5-dimethyl-4-nitrosopyrazole (2K5) and 1-*p*-chlorophenyl-3,5-dimethyl-4-nitrosopyrazole (J49). These nitrosopyrazoles were almost 100 times as active as tetrachlorobenzoquinone on slides and two to three times as effective as 2,3-dichloro-1,4-naphthoquinone on tomato foliage. No other group of nonmercurial organic compounds has been reported as possessing comparable fungitoxicity. If materials with this activity persist on foliage under field conditions, adequate disease control might be expected from a dosage of about 4 oz. or less per 100 gal. of spray mixture, since 12 to 16 oz. of Phygon will provide excellent control of apple scab and tomato leaf blight diseases.

Fungitoxicity of Reactants Used to Produce 4-Nitrosopyrazoles

Inasmuch as 4-nitrosopyrazoles are produced spontaneously in aqueous medium from a hydrazine (generally as the hydrochloride) and isonitrosoacetylacetone, it would seem unlikely that they could be hydrolyzed to regenerate the reactants. However, the possibility exists that the fungus could act as a hydrolytic agent and that one or both of the reactants account for the fungitoxicity of the 4-nitrosopyrazoles. The theory was sufficiently interesting to warrant testing; so an experiment was arranged wherein the compounds used to produce the 4-nitrosopyrazoles were tested alongside the appropriate reaction product.

Although many hydrazines were tested in the course of these studies, none of them were more fungitoxic than phenylhydrazine (E24); so it was chosen for these tests. When reacted with isonitrosoacetylacetone (P90), compound J55 is produced. Other hydrazines, α-naphthylhydrazine (16K) and *p*-chlorophenylhydrazine hydrochloride (5J0), and the nitrosopyrazoles (19K and J49) produced by reacting them with isonitrosoacetylacetone, were also included in these tests. There was not sufficient α-naphthylhydrazine available for the foliage tests in this series, but in previous tests it had proved to be relatively ineffective as a foliage protectant (87 per cent control at 2000 ppm and an LD95 $>$ 10,000 ppm).

Isonitrosoacetylacetone was practically nonfungitoxic even at dosages as heavy as 1000 ppm on glass slides and 2000 ppm on foliage (tables 3 and 4). Phenylhydrazine was fairly active on glass slides but was ineffective on tomato foliage. It was only a fraction as active in either test as the nitrosopyrazole (J55) produced by reacting it with isonitrosoacetylacetone.

The nitrosopyrazoles produced from α-naphthylhydrazine and *p*-chlorophenylhydrazine were 100 to 1000 times as effective as the parent hydrazine on glass slides. The data from tomato foliage tests provide an even better contrast. The three nitrosopyrazoles had LD95 values of 60 ppm (J55), 31.5 ppm (J49), and 30 ppm (19K). The two hydrazines used in synthesizing J55 and J49, respectively, had LD95 values of approximately 10,000 ppm (E24) and 3000 ppm (5J0).

The obvious conclusion from this test is that if the fungus or plant did hydrolyze the 4-nitrosopyrazole, the hydrolysis products would not be very fungicidal. The nitrosopyrazole must act as an entity. This is a logical assumption since they are relatively stable.

Chemotherapy and Solubility Experiments

The tomato foliage protection test is based upon the assumption that a uniform layer of fungitoxic chemical is deposited in position to prevent spore germination. Most of the good fungicides must be applied at concentrations of 200 to 600 ppm in order to provide 95 per cent control; so comparable control by the better 4-nitrosopyrazoles at concentrations of 10 to 60 ppm raises a question as to whether they operate in a conventional manner. They conceivably may operate in a molecular rather than particulate state as chemotherapeutic agents inside the leaves or as solutes on the leaf surface. Several of the more active compounds reported upon in table 8 were suspended in distilled water at 2000 ppm, employing Emulphor EL where necessary, and tested for solubility and chemotherapeutic activity by bioassay on tomato foliage.

Tests were made in triplicate on potted 7-in. plants. Three 30-ml. vials were filled with a 24-hr.-old suspension of each chemical, and the first fully expanded leaf on each plant was inserted in a vial. The vials were sealed with moisture-proof paper to prevent the chemical from reaching the sur-

face of adjacent leaves by splashing or volatilization. The plants were held in a greenhouse at 65° to 80° F. without watering for 36 hr. to permit uptake of chemical by the immersed leaf. About 8 ml. of suspension was absorbed before the leaf petiole was cut off and the retaining vial removed. The plants were watered, sprayed with a spore suspension of *Alternaria solani*, and placed in an incubation chamber for 24 hr. They were then removed to the greenhouse for 72 hr. before records were taken on the number of lesions.

The data in column 3 of table 9 show that J55 gave 44.4 per cent control

TABLE 9.—*Chemotherapeutic activity of several 1-(substituted phenyl)-3,5-dimethyl-4-nitrosopyrazoles*

Compound tested		Leaf blight chemotherapy	Control by filtrate spray	Estimated concentration in filtrate
Code	Substituent			
		Pct.	*Pct.*	*Ppm*
J55	None	44.4	88.6	37
0M6	*p*-Iodo	7.7	58.1	38
7K1	*p*-Bromo	21.3	63.7	49
4L9	*p*-Fluoro	22.6	88.1	27
J49	*p*-Chloro	24.0	32.7	5
35L	*m*-Chloro	11.6	75.8	12
27L	*o*-Chloro	18.8	71.7	9
3K4	2,4-Dichloro	24.4	87.2	61
3K3	2,5-Dichloro	24.3	87.4	60
2K5	*p*-Methyl	19.5	26.2	4
7K2	*p*-*tert*-Amyl	2.2	89.3	69
8K2	*p*-Nitro	5.5	84.0	210
39L	*p*-Thiocyano	5.4	66.6	76
36L	*p*-Sulfamyl	31.0	94.7	515
Min. Sign. Diff. at 5 per cent		9.6		
Min. Sign. Diff. at 1 per cent		17.0		

and 36L, 31 per cent; hence it must be deduced that some of the chemical was taken up by the immersed leaf and transported to the other leaves, where it provided appreciable protection by chemotherapy. Compound 36L was observed to be particularly effective in delaying the development of lesions. Counts made 40 hr. after inoculation showed 10 to 30 lesions per plant, while untreated plants had 250 to 350 lesions each. The relatively weak fungicides 7K2, 8K2, and 39L gave no significant control of blight.

These data on chemotherapy were not in all cases in close agreement with those from protectant tests (Table 8). The most obvious deviation was the unexpected superiority of 36L over J49, 2K5, and other superior protectants. These apparent inconsistencies were resolved by studies on the solubilities of the compounds. A 72-hr.-old suspension at a concentration of 2000 ppm was filtered through Whatman No. 5 paper, then passed through a fine sintered-glass filter. The filtrates probably contained some colloidal material in addition to the dissolved fraction, but a fair measure was obtained of the dissolved and fine fractions that could be easily drawn into the plant and circulated through its conductive tissue.

These filtrates were sprayed on duplicate plants, and data on blight control were obtained by the conventional method. The data in the fourth column of table 9 show 26 to 95 per cent disease control. Where these percentages were interpolated on the dosage-response curves obtained on comparable plants at the same time (Table 8), estimates were obtained of the actual concentrations of active ingredients. These estimates, presented in the last column of table 9, show that the concentrations of the most highly fungitoxic compounds in the filtrates probably were not greater than 4 ppm for 2K5, 5 ppm for J49, 9 ppm for 27L, and 12 ppm for 35L. Compound 36L, which is not exceptionally fungitoxic, probably achieved its excellent chemotherapeutic performance because of its greater solubility. Compound J55, which excelled 36L in chemotherapeutic protection, is much less soluble, but the dissolved material probably is about eight times as potent (Table 8) and hence much more effective at low concentrations. On the other hand, J55 is so much more soluble than the superior fungicides that it has an opportunity to be taken into the plant in sufficient quantity to provide systemic protection.

These tests show that J49 and 2K5 have the characteristics essential for a good foliage protectant. They combine strong fungitoxicity with minimum solubility; so they should be retained on the foliage at effective concentrations during rainstorms. Because of this, these two compounds were used for extensive field trials as seed and foliage protectants.

Value of 4-Nitrosopyrazoles as Pea Seed Protectants

Several nitrosopyrazoles were tested for ability to protect Thos. Laxton and Perfection pea seed in the greenhouse and field. The effect of various substituents in the 1-position of 3,5-dimethyl-4-nitrosopyrazole was determined in two tests made in the greenhouse on different dates with the seven compounds listed in table 10. Seed treated with each chemical at three dosage levels was sown in naturally infested soil. In the first test the soil was moderately moist, while in the second it was watered heavily so that seed decay would be more severe. Spergon was included in the test as a standard, since it is widely used on pea seed.

The only injurious material was the soluble 1,3,5-trimethyl-4-nitrosopyrazole (4K3) which gave significant improvement in emergence at the extremely low dosage of 0.02 per cent but suppressed germination at higher dosages. Compounds 7K2 and 3K4 were somewhat less effective than the other nitrosopyrazoles, a situation anticipated from their fungitoxicity records. The other compounds, particularly J55, J49, and 2K5, were fully as effective as Spergon at comparable dosages. In general, the seed protectant ability of these compounds agreed closely with their fungitoxicity as shown in tables 7 and 8.

Comparable confirmatory data were obtained on 0M6, 4L9, and L67 in another greenhouse test made on Thos. Laxton pea seed. The mean emer-

TABLE 10.—*Effectiveness of several 3,5-dimethyl-4-nitrosopyrazoles with various substituents in the 1-position as pea seed protectants in severely infested greenhouse soil*

Treatment applied to seed			Emergence of plants	
Code	Substituent	Dose	Test 1	Test 2
		Pct.	*Pct.*	*Pct.*
J49	*p*-Chlorophenyl	0.46	82.0	66.0
J49	*p*-Chlorophenyl	0.09	71.0	54.5
J49	*p*-Chlorophenyl	0.02	70.0	47.5
J55	Phenyl	0.55	82.5	73.5
J55	Phenyl	0.13	80.5	58.5
J55	Phenyl	0.03	71.0	51.0
7K2	*p-tert*-Amylphenyl	0.54	70.0	47.0
7K2	*p-tert*-Amylphenyl	0.13	63.5	45.0
7K2	*p-tert*-Amylphenyl	0.02	64.5	45.0
7K1	*p*-Bromophenyl	1.05	73.5	61.5
7K1	*p*-Bromophenyl	0.25	70.0	63.0
7K1	*p*-Bromophenyl	0.10	68.0	50.5
2K5	*p*-Tolyl	0.51	81.0	64.5
2K5	*p*-Tolyl	0.14	75.5	61.0
2K5	*p*-Tolyl	0.04	72.0	51.5
3K4	2,4-Dichlorophenyl	0.59	63.5	61.0
3K4	2,4-Dichlorophenyl	0 09	62.5	49.0
3K4	2,4-Dichlorophenyl	0.01	64.0	44.0
4K3	Methyl	0.28	39.0	22.5
4K3	Methyl	0.17	64.5	40.0
4K3	Methyl	0.02	73.5	52.0
Spergon		0.78	80.0	62.5
Spergon		0.16	77.0	56.0
Spergon		0.01	71.0	47.5
None		.....	59.5	35.5
None		.....	66.5	33.5
None		.....	62.5	37.5
None		.....	61.5	30.5
Min. Sign. Diff. at 5 per cent		.....	9.9	11.6
Min. Sign. Diff. at 1 per cent		.....	13.1	15.2

gence in eight replications of 25 seeds each was: 14 and 23.5 per cent in the untreated controls; 87.5, 83.5, and 68 per cent for Spergon-treated seed at 0.43, 0.20, and 0.10 per cent by weight; 80.5, 70.0, and 48 per cent for 4L9 (1-*p*-fluorophenyl-3,5-dimethyl-4-nitrosopyrazole) at 0.17, 0.10, and 0.03 per cent; 67.5, 60.5, and 57.5 per cent for 0M6 (1-*p*-iodophenyl-3,5-dimethyl-4-nitrosopyrazole) at 0.14, 0.03, and 0.01 per cent. These data show the 1-*p*-fluorophenyl derivative to be as effective as Spergon and superior to the analogous 1-*p*-iodophenyl compound. In this test seed treated with L67 (1-phenyl-3,5-dimethyl-4-nitropyrazole) produced 39, 35, and 30 per cent emergence at dosages of 0.20, 0.09, and 0.04 per cent by weight. The results were to be anticipated from the relatively weak fungitoxicity exhibited by this compound both on glass slides and tomato foliage.

Another greenhouse test was made with pea seed of the variety Thos. Laxton to determine the relative effectiveness of compounds containing different substituents on the 1-phenyl group. The data presented on 27L, 35L, 36L, 39L, and Spergon in table 11 show all of these materials to be excel-

TABLE 11.—*Relative effectiveness of 3,5-dimethyl-4-nitrosopyrazoles as pea seed protectants under greenhouse conditions*

Treatment applied		Emergence from seed with dose of		
Code	Substituent in 1-position	0.20 per cent	0.10 per cent	0.05 per cent
		Pct.	*Pct.*	*Pct.*
27L	*o*-Chlorophenyl	68.0	66.0	49.0
35L	*m*-Chlorophenyl	68.5	57.0	52.5
36L	*p*-Sulfamylphenyl	85.5	76.0	60.5
39L	*p*-Thiocyanophenyl	65.5	58.5	49.0
Spergon		77.0	59.0	57.5
None		34.0	38.5	
Min. Sign. Diff. at 5 per cent				18.0
Min. Sign. Diff. at 1 per cent				24.0

lent seed protectants. The outstanding member is 36L (1-*p*-sulfamylphenyl-3,5-dimethyl-4-nitrosopyrazole), a compound derived from sulfanilamide. It is surprising that this material proved to be fully as effective as Spergon since it was not outstanding on glass slides (Table 7). The question may be raised as to whether its chemotherapeutic activity and water solubility (Table 9) may not have given it an advantage in this trial.

The effectiveness of 36L was amply confirmed in a field trial. The mean emergence in five replications of 100 seeds each treated with 36L at dosages of 0.30, 0.15, and 0.07 per cent by weight was 86.2, 89.3, and 87.8 per cent, respectively, as compared to 88.8, 90.5, and 79.7 per cent for Spergon at comparable dosages, and 69.2, 68.8, and 71.0 per cent for untreated controls. In this test the minimum significant difference at the 5 per cent point was 8 per cent; at the 1 per cent point, 11 per cent. These data were confirmed in 1947 and 1948 by other tests made in the greenhouse and field on seeds of peas, beets, and sweet corn.

Some of the compounds evaluated under greenhouse conditions were given more extensive trials in one greenhouse and two field plantings made during the 1946 season. The compounds used had different substituents in the 1-position of 3,5-dimethyl-4-nitrosopyrazole. All materials except 2K5 which was diluted in talc were applied as concentrated dusts. Each formulation was applied at dosages of approximately 0.64, 0.16, and 0.04 per cent by weight of the pea seed. The mean emergence obtained from 5 replications of 100 seeds each in the first field trial at each of these dosages was as follows: 2K5 (*p*-tolyl derivative) at 100 per cent concentration 79.8, 86.4, and 79.4; 2K5 at 50 per cent concentration 82.0, 72.6, 74.8; 2K5 at 25 per cent concentration 76.4, 84.0, 68.2; J49 (*p*-chlorophenyl derivative) 82.0, 82.8, 80.4; J55 (phenyl derivative) 75.8, 68.2, 77.8; 7K1 (*p*-bromophenyl derivative) 85.2, 89.0, 78.0; 7K2 (*p-tert*-amylphenyl derivative) 84.2, 67.6, 72.6; tetrachloro-*p*-benzoquinone and DDT 85.6, 83.0, 75.4; and untreated controls 74.0, 68.0, and 78.8 per cent, respectively. The minimum significant differences at the 5 and 1 per cent points were 13.6 and 17.9 per cent, respectively.

In the second field trial the mean emergence from seed bearing the same dosages of the materials as listed in the above order was: 89.6, 86.4, and 80.8; 81.2, 81.0, and 76.8; 82.4, 76.8, and 74.4; 88.4, 81.0, and 81.0; 84.8, 80.6, and 76.2; 88.0, 84.4, and 79.8; 82.0, 78.6, and 73.4; 86.0, 86.0, and 80.5; and 71.6, 70.0, and 69.0. The minimum significant difference between means was 6.4 at the 5 per cent level and 8.4 at the 1 per cent level.

Slightly more severe conditions prevailed in the trial made in the greenhouse, but comparable results were obtained. The corresponding emergence from seed treated with the materials listed above was: 89.6, 88.0, and 72.0; 87.2, 80.0, and 68.0; 83.2, 77.6, and 54.4; 80.8, 88.0, and 80.0; 88.0, 90.4, and 76.8; 80.8, 86.4, and 70.4; 68.8, 64.8, and 60.8; 88.0, 87.2, and 70.4; and 61.6, 60.8, and 62.4, respectively. The minimum significant differences between means in this test were 5.7 and 7.6 per cent at the 5 and 1 per cent levels.

The data from these three experiments are in close agreement. The better nitrosopyrazoles compared favorably with Spergon. Compound J55 did not prove superior either to 2K5 or J49; so it is concluded that the small differences in its favor in preceding tests (Table 10) were entirely attributable to experimental error. Compound 7K2 continued to provide poor seed protection under conditions favorable to seed decay. When 2K5 was diluted with talc, its effectiveness was reduced. It may be concluded that the material is approximately twice as effective as Spergon, since the 50 per cent concentration gave results analogous to those of Spergon at full strength.

The Effectiveness of Two 4-Nitrosopyrazoles as Seed Protectants for Corn, Spinach, and Beets

Two of the effective pea seed protectants, J49 and 2K5, were tested in field trials as protectants for sweet corn seed of the variety Golden Cross Bantam (Table 12). Two experiments were made employing five replications of 100 seeds each from each dosage. In the first test, repeated heavy rains and 10 cold days immediately after planting created ideal conditions for seed decay. Only 33 per cent of the untreated seed produced plants and these were small and weak. Over 73 per cent of the Phygon-treated seed produced plants. Under the conditions of this experiment this material would equal or surpass other commercial products. Compound 2K5 was about as effective as Phygon at the two higher dosages but did not give comparable protection at the lowest dosage. However, its performance would definitely classify it as a commercial possibility.

Compound J49 was less effective than 2K5 at all dosage levels. However, it was definitely a better protectant than Semesan Jr. which was used as a standard of comparison. Under the more moderate disease conditions prevailing in the second test, J49 was as satisfactory as the other two materials. This test was not sufficiently severe to provide a true measure of fungicidal efficiency, since there was little difference among either the materials or the various dosages.

TABLE 12.—*Relative effectiveness of two 4-nitrosopyrazoles and standard fungicides as sweet corn seed protectants under field conditions*

Treatment applied		Seedling emergence	
Material	Dose	Test 1	Test 2
	Pct.	*Pct.*	*Pct.*
Phygon	0.54	73.2	67.4
Phygon	0.20	74.0	70.6
Phygon	0.07	72.8	65.6
2K5	0.52	72.4	73.4
2K5	0.18	70.0	71.2
2K5	0.07	54.0	69.4
J49	0.54	64.6	74.0
J49	0.19	61.2	69.2
J49	0.07	47.4	70.6
Semesan Jr.	0.19	45.0	71.0
None	.....	32.2	64.4
None	.....	33.4	65.0
None	.....	33.0	64.2
Min. Sign. Diff. at 5 per cent		7.3	5.6
Min. Sign. Diff. at 1 per cent		9.7	7.5

The same two nitrosopyrazoles were tested on spinach seed of the variety Virginia Savoy at three dosage levels in a series of greenhouse and field trials (Table 13). Phygon and Phygon-DDT were used as standards

TABLE 13.—*Relative effectiveness of nitrosopyrazoles and standard fungicides as spinach and beet seed protectants under field and greenhouse conditions*

Treatment applied to seed		Emergence from 100 seed units			
		Spinach		Beets	
Material	Dose	Field	G.H.	Field	G.H.
	Pct.	*Pct.*	*Pct.*	*Pct.*	*Pct.*
Phygon	2.00	60.6	78.0	58.3	110.5
Phygon	1.00	66.6	81.6	58.8	107.8
Phygon	0.50	68.4	76.0	71.9	100.2
Phygon-DDT	2.00	69.0	79.6	65.8	100.2
Phygon-DDT	1.00	65.8	80.4	71.9	99.2
Phygon-DDT	0.50	61.2	81.2	68.8	95.2
2K5 (100 per cent)	2.00	63.0	74.0	63.5	99.5
2K5 (100 per cent)	1.00	61.4	76.0	63.5	105.0
2K5 (100 per cent)	0.50	53.0	74.4	61.5	81.8
2K5 (25 per cent)	2.00	56.0	78.0	66.0	84.0
2K5 (25 per cent)	1.00	59.4	73.2	54.3	69.5
2K5 (25 per cent)	0.50	45.6	76.4	42.5	55.8
J49 (100 per cent)	2.00	47.2	72.4	71.0	100.8
J49 (100 per cent)	1.00	52.4	78.4	66.5	72.5
J49 (100 per cent)	0.50	40.4	66.4	54.3	54.5
None	.....	23.2	27.6	13.0	9.2
None	.....	22.8	25.6	15.3	9.2
None	.....	24.0	23.6	18.1	7.8
Min. Sign. Diff. at 5 per cent		9.2	8.7	7.3	24.0
Min. Sign. Diff. at 1 per cent		12.2	11.5	9.6	31.8

since they were known to be exceptionally effective on this crop. The data obtained agree fairly well with those obtained on corn seed. Under field conditions the 2,3-dichloro-1,4-naphthoquinone formulations were slightly more effective than 2K5 at the lowest dosage but not significantly different at the higher dosages. The initial stand obtained in the greenhouse showed 2K5 to be equal to Phygon when used at 100 per cent concentration. When 2K5 was diluted to 25 per cent with talc, it performed as well as the other materials in the greenhouse but was less effective under field conditions. Compound J49 was slightly (but not significantly) less effective than 2K5 under greenhouse conditions and was definitely less effective in the field trial.

Tests similar to those on spinach were made on beet seed of the variety Asgrow Canner (Table 13). The average emergence in eight replications of 100 seed balls each in the first field trial showed that 2K5 was slightly more effective (but not significantly so) than Phygon. In a second field trial there was no appreciable difference between the two treatments at the higher dosages, but Phygon was superior at the lowest dosage employed. Under greenhouse conditions the materials again gave approximately the same results. Compound 2K5 was also very effective when used as a 25 per cent dust in talc at dosages of 1 to 2 per cent by weight of seed.

Compound J49 provided good seed protection when used at a dosage of 2 per cent. At lower dosages it gave mediocre protection. It should be noted that all of these tests were under conditions extremely favorable to seed decay, and even the least effective dosage improved emergence by 50 to 300 per cent.

Value as Foliage Protectants

On the basis of their ability to protect tomato foliage in the greenhouse, J49 and 2K5 were selected as the most promising of the nitrosopyrazoles for testing as foliage protectants in field trials. In addition to being active fungicides, they were relatively insoluble and without injurious effects when sprayed on tomato foliage at concentrations as high as 3000 ppm. The practical dosage was estimated to be 4 oz. or less per 100 gal. of spray; so a dosage series of 8, 4, 2, and 1 oz. was selected for apple spray tests.

The treatments were applied at these four dosages to single-tree replicates on the varieties McIntosh, Rome Beauty, Northern Spy, and Baldwin in an orchard about 20 years of age. The 1946 season was extremely favorable to the development of scab (*Venturia inaequalis* (Cke.) Aderh.) and rust (*Gymnosporangium juniperi-virginianae* Schw.). Two prebloom, a calyx, and four cover sprays were applied (April 22, May 1 and 20, June 3 and 17, July 2 and 26). During the 19-day interval preceding the calyx spray a series of five heavy rains and continuous cloudy weather for a week created ideal conditions for spread of conidia produced from primary infection. The schedule followed was obviously inadequate for such a season, and the less effective materials permitted serious foliage infection.

Records were taken on June 26 of scab infection on leaves at six loca-

tions on each tree at 4 to 7 ft. above the ground level. The data on the variety McIntosh presented in table 14 show that J49 provided as good protec-

TABLE 14.—*Protection of apple fruit and foliage from scab and rust by J49 and other fungicides—1946*

Spray treatment		Amount of scab infection			Cedar rust	
Material applied	Dose/100 gal.	On leaves June 26	Lesions per fruit	Commercial control	Lesions per leaf	Disease control
	Oz.	*Pct.*	*No.*	*Pct.*	*No.*	*Pct.*
Phygon, wet.	32	5	0.00	100.0	0.097	93.6
Phygon, wet.	16	2	0.80	89.6	0.357	76.5
Phygon, wet.	8	3	0.25	98.8	0.462	69.5
Phygon-Sulfur[a]	32	1	0.02	100.0	0.429	71.7
Phygon-Sulfur[a]	16	6	0.01	100.0	0.443	70.8
Phygon-Sulfur[a]	8	14	0.51	99.7	0.800	47.2
J49	8	6	0.10	99.7	1.386	8.6
J49	4	5	0.29	99.3	1.110	26.8
J49	2	44	46.30	39.6	1.670	0.0
J49	1	29	44.06	33.4	1.286	15.2
Lime-Sulfur	(1.5 gal.)	6	0.11	93.9	0.719	52.6
Wettable Sulfur	128	70	7.29	74.3	.....	.....
None		75	23.47	44.9	1.516	.....

[a] Phygon through calyx; wettable sulfur 8 lb. per 100 gal. in cover sprays.

tion at dosages of 8 and 4 oz. as lime-sulfur at 1.5 gal. per 100 gal., and was only slightly less effective than Phygon at the higher dosages. It was decisively superior to wettable sulfur. There was a sharp break in disease control by J49 between dosages of 4 and 2 oz. per 100 gal. Comparable data were obtained on foliage protection of Rome Beauty and Northern Spy.

As the fruit was harvested from each tree it was classified according to the presence of commercial scab; and 2 bushels of fruit, one from the upper branches and the other from the lower ones, were examined for total number of lesions. The data in table 14 show that 45 per cent of the unsprayed fruit escaped severe infection but that there was an average of 23.47 lesions per fruit. The data on fruit infection followed the trend observed on foliage with J49 providing remarkable control at dosages of 8 and 4 oz. but failing at lower rates.

The data in table 14 should be taken as a strong indication of relative merits rather than as complete proof because of the lack of replication of each dosage on the various varieties. The data demonstrated, however, that J49 used at a dosage of 4 oz. per 100 gal. provided as good scab control as lime-sulfur at 1.5 gal. The large number of lesions on fruit sprayed with J49 at low dosages is unexplained. In the latter part of July, many small superficial scab lesions developed uniformly on these trees. These multiplied in number even though few of them caused large lesions or cracking of the fruit.

The orchard was bordered on two sides by natural vegetation which included many rust-infected red cedars. The variety Rome Beauty became

severely infected with rust during the first two weeks of May. At the time the foliage was being examined for scab, the number of rust lesions on this variety was counted. The data summarized in the last two columns of table 14 leave little doubt that J49 was as weak against rust as it was effective against scab. It failed to equal lime-sulfur in rust control. Phygon was definitely superior to both materials.

There was no evidence of foliage injury from J49 at these dosages or at the rate of 1 lb. per 100 gal. applied to three trees outside this experiment. The fruits of McIntosh, Rome Beauty, and Northern Spy were not injured, but Baldwin fruit had appreciable russet at dosages as low as 2 oz. per 100 gal.

Compounds J49 and 2K5 were applied in dosage series to single-row plots of John Baer tomatoes and Green Mountain potatoes. The materials were applied on July 30 and August 9, 22, and 30. This schedule was undoubtedly too abbreviated for the 1946 season. At least one more application could have been used to advantage on tomatoes and three more on potatoes. The season was cool and frequent rains encouraged the development of *Phytophthora infestans* (Mont.) deB. The disease was very prevalent on unsprayed plants by August 15 and most of the foliage was dead by September 15. Severe fruit rot was observed on the tomatoes by the latter part of August, and practically no marketable fruit was harvested from the controls after the first picking.

Both of the nitrosopyrazoles provided considerable protection, as can be seen from the records on blight control in table 15, taken by the visual scor-

TABLE 15.—*Effectiveness of nitrosopyrazoles in preventing Phytophthora blight of tomato and potato*

Spray treatment		Tomato trials		Potato trials	
Material applied	Dose/100 gal.	Blight control	Marketable fruit[a]	Blight control	Yield of tubers[b]
	Oz.	*Pct.*	*Lb.*	*Pct.*	*Lb.*
2K5	16	.....	.....	64	18.6
2K5	8	84	12.83	82	17.4
2K5	4	80	10.73	64	12.9
2K5	2	65	10.49	25	11.6
J49	16	.....	.....	79	16.9
J49	8	76	10.67	58	13.7
J49	4	77	7.95	43	14.3
J49	2	69	9.35	50	11.6
Phygon, wet.	32	94	43.49	94	24.3
Phygon, wet.	16	92	32.82	93	18.9
Phygon, wet.	8	76	20.13	85	19.4
Phygon, wet.	4	.....	.....	81	16.1
None	.....	35	7.4	9	13.3
Min. Sign. Diff. at 5 per cent		.....	6.67	.....	6.3
Min. Sign. Diff. at 1 per cent		.....	8.86	.....	8.3

[a] Yield from 7 plants.
[b] Yield from 25 plants.

ing method. The control was not considered as commercially acceptable and was definitely inferior to that obtained from Phygon. These differences were accentuated later in the season, and the plants sprayed with the nitrosopyrazoles were practically defoliated by late September. This failure to control blight was reflected in the yields of both crops. There was a moderate increase from their use but it was not so large as that obtained from Phygon.

The reason for this partial failure of the nitrosopyrazoles is not immediately clear. The dosage level may have been too low to control the disease in a year of extremely severe infestation, or the reduced spray schedule may have been responsible. It is also possible that this type of fungicide is ineffective against *Phytophthora infestans* in the same fashion as it failed against cedar-apple rust and as certain other fungicides such as sulfur and metallic dithiocarbamates fail to control late blight. This latter possibility is considered to be the most probable explanation.

Dermatological Effects

The various nitrosopyrazoles were used throughout the series of laboratory and greenhouse tests without observing any adverse effects on the experimenters. In conducting orchard spray tests with J49 and 2K5, the operator noticed a moderate irritation immediately beneath his eyes but it was not considered serious. Appreciable irritation did occur in one test when the sleeve of the operator's coveralls became saturated with spray. His wrist was sensitized for about 10 hr. at the point where the sleeve rubbed against it.

Although the materials did not cause serious dermatitis to the spray operators, they did cause a rash on factory operators who dried and ground large samples for field evaluation. It was considered desirable, therefore, to conduct dermatological tests on 2K5, J49, and other pyrazoles. Thirteen compounds were chosen for tests on white rabbits. Undiluted chemical was rubbed into an area behind the shoulders that had been shaved two days previously. The material was sealed into place by covering it with lanolin. This is an unusually severe method of testing but it has the advantage of detecting weakly irritant materials.

The data in table 16 show that the best fungicides (2K5 and J49) were among the more irritant and the relatively poor fungicide 7K2 (with a *p-tert*-amylphenyl group in the 1-position) was relatively nonirritant. With other substituents in the *para* position of the phenyl group, irritant effects between these extremes were observed. The fluorine derivative was the safest of the halogen-containing compounds. The compound with an unsubstituted phenyl group (J55) was one of the more irritant. The replacement of the phenyl group in the 1-position by a hydrogen atom appreciably reduced the irritant effect but did not completely eliminate it.

Since the nitroso group is generally recognized as causing dermatological

TABLE 16.—*Dermatological effects on rabbits of 3,5-dimethylpyrazoles containing various substituents in the 1- and 4-positions*

Code	Substituent in		Condition of skin after	
	1-position	4-position	24 hours	168 hours[a]
J49	*p*-Chlorophenyl	Nitroso	Intense red, swollen	Dry, red, sloughing
2K5	*p*-Tolyl	Nitroso	Intense red, swollen	Red, swollen
J55	Phenyl	Nitroso	Red, swollen	Very red, sloughing
4K3	Methyl	Nitroso	Intense red	Red, swollen
8K2	*p*-Nitrophenyl	Nitroso	Red, swollen, sensitive	Red, swollen
7K1	*p*-Bromophenyl	Nitroso	Red, swollen, sensitive	Red, sloughing
19K	α-Naphthyl	Nitroso	Red, not sensitive	Red
30J	Hydrogen	Nitroso	Slight red, not sensitive	Slight red
J54	*p*-Chlorophenyl	Hydrogen	Red, swollen, sensitive	Normal, hair growing
7K2	*p*-*tert*-Amylphenyl	Nitroso	Red, not sensitive	Normal
4L9	*p*-Fluorophenyl	Nitroso	Red, not sensitive	Normal, hair growing
3K3	2,5-Dichlorophenyl	Nitroso	Normal	Normal, hair growing
3K4	2,4-Dichlorophenyl	Nitroso	Trace of red	Normal, hair growing
Lanolin control			Normal	Normal

[a] None of the treated areas seemed sensitive but some edema was evident in treatments 4K3, 2K5, and 8K2.

effects, compound J54, with a hydrogen atom instead of a nitroso group in the 4-position, was included in the test. This material had preliminary irritant properties comparable to the nitroso derivative, but the animal recovered more rapidly.

Of particular interest was the mild reaction incited by 3K3 and 3K4, the 1-(dichlorophenyl) derivatives. These materials, which are excellent fungicides, caused much less dermatitis than any of the other nitrosopyrazoles in this test. They were next subjected to patch tests in undiluted form on the forearm of humans. Some individuals showed very little reaction, but others had pronounced reddening and swelling beneath the patch.

In general, these compounds show dermatological effects in proportion to their fungicidal activity. There are enough exceptions to this rule, however, to offer some encouragement. The fact that such good fungicides as 3K3, 3K4, and 19K are moderately safe provides room for development of safe, effective fungicides.

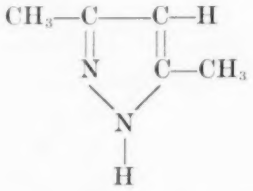
In another test made on rabbits, 36L, after its ability to protect seed had stimulated interest in it, was found to be nonirritant. There was no swelling or reddening after 2 days and the hair was growing back normally on the 8th day after treatment.

It may be concluded that there is a tendency for the 4-nitrosopyrazoles to irritate when maintained in intimate contact with the skin for prolonged

periods. It is possible, however, to modify the substituent in the 1-position so as to eliminate much, if not all, of this irritant effect.

DISCUSSION

Most of the compounds discussed in this paper are related to 3,5-dimethylpyrazole (1E1):



It was a relatively weak fungicide both in glass-slide and tomato-foliage tests. By substituting a nitroso (–NO) group in the 4-position, a moderately active compound (30J) was obtained. Comparable activation could not be obtained by substituting other groups such as nitro ($-NO_2$), amino ($-NH_2$) or phenylazo ($-N=NC_6H_5$) in this position. Compound 1E1 was activated slightly by substitution of a *p*-chlorophenyl group in the 1-position to produce J54. The full benefit of substitution in this position, however, was realized only on those pyrazoles containing a 4-nitroso group. The effective groups in the 1-position, in descending order of activation, were α-naphthyl, phenyl, methyl, and hydrogen. The effectiveness of the 1-phenyl group was further enhanced by substitution of a chlorine atom or methyl group in the *para* position to produce J49 and 2K5. The phenyl group did not exert a comparable effect when in the 5-position.

The methyl groups in the 3- and 5-positions occur as an inevitable result of the method of synthesis used. They are not considered as strong contributing forces to the fungitoxicity of the compounds. It was found, however, that the replacement of one of the methyl groups by a longer carbon chain, a *n*-hexyl group, resulted in a less effective fungicide.

The pyrazole nucleus cannot be altered appreciably without loss of fungitoxicity. When the nitroso group was substituted in the 4-position of a 3-pyrazolone as in 49K instead of in the 4-position of a pyrazole, a very weak fungicide was produced. Both the nitroso group and the pyrazole nucleus are essential for fungicidal effectiveness.

The compounds used in synthesizing these potent fungicides are relatively inactive. Isonitrosoacetylacetone was practically inert at the dosages employed in these tests, and the various hydrazines were only weakly fungitoxic.

None of the data obtained provides a suggestion as to why the 4-nitrosopyrazoles are fungitoxic. Insofar as can be determined, pyrazoles do not occur in nature (11); so there are no known analogs or comparable structures that might be blocked as reported by Woods (32) for the blocking of *p*-aminobenzoic acid by sulfanilamide. Neither is there reason to assume

that they prevent carbohydrate metabolism by converting reducing sugars into osazones, as might occur if they hydrolyzed to a hydrazine and isonitrosoacetylacetone. All of the available evidence suggests that the nitrosopyrazoles act as a unit. The nitrosopyrazoles may operate by interfering with reversible oxidation-reduction systems within a fungus cell. They, like many other good fungicides such as sulfur and quinones, are strong oxidants. More data are required, however, before drawing conclusions.

The more effective members of the 4-nitrosopyrazole series probably are the most active organic fungicides revealed to date. There are a dozen members that perform as well in laboratory and greenhouse tests as standard fungicides. Three members (J49, 2K5, 19K) are two to three times as effective in protecting tomato foliage as 2,3-dichloro-1,4-naphthoquinone, a material that controls apple scab at a dosage of 0.75 lb. per 100 gal. Their performance would classify them as superior in toxicity to other nitrogenous fungicides such as the quaternary ammonium (10, 16), glyoxalidine (29), 8-hydroxyquinoline (24), and N-nitrosophthalimidine (15) compounds. It is interesting to note that the glyoxalidines and pyrazoles have certain similarities. Both contain five-membered heterocyclic rings with two nitrogen atoms; but a carbon atom occurs between the two nitrogens in the glyoxalidine nucleus. A long-chain alkyl group is required to activate the glyoxalidines (29), and specific groups must be substituted on the pyrazoles to attain maximum fungitoxicity.

In a series of greenhouse and field trials, excellent seed protection was obtained from use of 36L, 2K5, J49, and other members of the series. The performance of these materials on seed of peas, maize, spinach, and beets was fully equal to that of standard commercial seed treatment materials. In general, the performance of 4-nitrosopyrazoles as seed protectants was in keeping with their fungicidal activity on glass slides and tomato foliage. However, one compound, 1-*p*-sulfamylphenyl-3,5-dimethyl-4-nitrosopyrazole (36L), which provided exceptional protection to seed, had given weak inhibition of spores on glass slides and only moderate control of leaf blight on tomatoes.

As a foliage spray, J49 gave almost perfect control of apple scab at a dosage of 4 oz. per 100 gal. It failed, however, to control cedar-apple rust and was only partially effective against *Phytophthora infestans* on potatoes and tomatoes.

The 4-nitrosopyrazoles are considered as having considerable commercial possibilities. Their greatest handicaps, from the information available, are a tendency to cause dermatitis, and a moderately high manufacturing cost.

SUMMARY

By analysis of the relations of chemical structure to fungitoxic action, it has been possible to create a remarkably effective group of new fungicides, the 4-nitrosopyrazoles. Maximum efficiency was attained by substituting an aryl group in the 1-position of 3,5-dimethyl-4-nitrosopyrazole.

The more active members of the series prevent spore germination on glass slides at concentrations as low as 0.1 ppm; provide 95 per cent protection against *Alternaria solani* on tomato foliage at concentrations of 10 to 60 ppm in spray suspension; give commercial control of apple scab at a concentration of 4 oz. per 100 gal. of spray mixture; and match, or excel, the performance of standard commercial fungicides in protecting seed of peas, maize, spinach, and beets from seed-decay and damping-off organisms in the soil.

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RELATIVE ABUNDANCE OF PHYTOPHTHORA CINNAMOMI IN THE ROOT ZONES OF HEALTHY AND LITTLE-LEAF-DISEASED SHORTLEAF PINE[1]

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During the spring and summer of 1948, *Phytophthora cinnamomi* Rands was isolated from the fine roots of littleleaf-diseased shortleaf pine (*Pinus echinata* Mill.).[2] Later in the year the development of a method for assaying soil for the presence of *P. cinnamomi* made it possible to determine the relative abundance of this fungus in the root zones of both healthy and littleleaf-diseased trees.[3] By this method, which involves the use of apples for the selective isolation of *P. cinnamomi*, soil was assayed under 20 healthy trees on littleleaf-free sites, 20 healthy trees on littleleaf sites, and 20 trees with typical or advanced littleleaf. The results are given in table 1.

TABLE 1.—*Relative abundance of Phytophthora cinnamomi in the root zones of healthy and littleleaf-diseased shortleaf pine*

Tree condition	Stand condition	Total trees sampled	Soil samples per tree	Soil samples yielding *P. cinnamomi*—average per tree	
		Number	*Number*	*Number*	*Per cent*[a]
Healthy	Healthy	20	24	1.1[b]	4.6
Healthy	Littleleaf	20	24	3.5[c]	14.6
Littleleaf	Littleleaf	20	24	10.0[d]	41.7

[a] The difference between each pair of percentages is statistically highly significant by the Chi Square Test.
[b] Range 0 to 4 per tree. No Phytophthora was isolated from the soil under 10 trees.
[c] Range 0 to 8 per tree. No Phytophthora was isolated from the soil under 5 trees.
[d] Range 4 to 18 per tree.

Soil samples were taken during December, 1948, and January and February, 1949. The weather during these months was comparatively mild, with only two periods of short duration when the minimum temperatures were much below freezing. *P. cinnamomi* was readily isolated from soil samples during the entire 3-month period. Soil samples from all types of stand and tree condition were taken concomitantly so that weather conditions that might influence isolations would be reflected in both healthy and diseased material.

Twenty-four soil samples were taken under each tree; each to a depth of 5 to 6 in. after the top inch of litter and humus had been cleared away.

[1] The work was done under the Division of Forest Pathology, Bureau of Plant Industry, Soils, and Agricultural Engineering, U. S. Department of Agriculture, in cooperation with the School of Forestry, University of Georgia, Athens, Georgia.

[2] Campbell, W. A. *Phytophthora cinnamomi* associated with the roots of littleleaf-diseased shortleaf pine. U. S. Dept. Agr., Pl. Dis. Reptr. 32: 472. 1948.

[3] Campbell, W. A. A method of isolating *Phytophthora cinnamomi* directly from soil. U. S. Dept. Agr., Pl. Dis. Reptr. 33: 134–135. 1949.

These samples were taken at 2-ft. intervals starting from the base of the tree; six in the direction of each cardinal compass point.

Selections of healthy trees from healthy stands were made within a radius of 20 miles north, east, and west of Athens, Georgia. These stands ranged in age from 60 to 100 years. Selections of healthy and littleleaf-diseased trees from littleleaf stands were made from areas near Athens, Flowery Branch, Carnesville, and Elberton, Ga., and Union, Walhalla, and Abbeville, S. C. These trees ranged from 20 to 60 years of age.

The data obtained in this study demonstrate that *P. cinnamomi* is present to a limited extent in the soil around healthy trees in stands that are free of littleleaf but that it is much more abundant in the root zone of littleleaf trees. This is considered strong evidence that *P. cinnamomi* is involved in the fine root necrosis that is known to accompany the disease.[4]

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FUSARIUM WILT OF STAGHORN SUMAC

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Staghorn sumac (*Rhus typhina* L.) is a woody shrub that occurs widely in the Northeast, west through Ontario to eastern North Dakota, and south along the Appalachian range to Alabama. It is a source of tannin, an important game food plant, and is valued as an ornamental because of its brilliant fall coloring and its crimson fruit clusters that persist through the winter.

During the summer of 1946, patches of wilting and dead sumac were found along the Blue Ridge Parkway, near Waynesboro, Virginia. Field examination showed that numerous sumac clumps were affected in an area of about 5 acres. A *Fusarium* was consistently isolated from discolored wood of diseased material. In 1947, many more sumac plants were found affected in the original area, and scattered affected shrubs were observed up to about 3 miles north and 7 miles south from this point. A brief report of this disease was made before the 1948 annual meeting of the Southern Division of The American Phytopathological Society,[2] and a description of the causal fungus, *Fusarium oxysporum* forma *rhois* Snyder and Hepting, and its behavior in culture has been made by Snyder *et al.*[3] Scouting in 1948 disclosed a few wilted plants a few miles north of those reported in 1947, but within the wilt area no appreciable spread of the disease had taken place since 1947. In the fall of 1948, Hepting observed staghorn sumac plants with wilt symptoms in New Haven, Connecticut. Typical cultures of *F. oxysporum* f. *rhois* were isolated from the discolored vascular systems and from sporodochia fruiting on some of the diseased stems. This disease has not been reported from any other localities to date or on any other species of *Rhus*. The present paper describes the symptoms and etiology of the Fusarium wilt of staghorn sumac.

SYMPTOMS

The first external symptom of the disease is wilting of some of the leaves. The wilted leaves hang sharply downward from the twigs (Fig. 1, A) and may remain attached to the stem for some time, making it easy to detect diseased plants from a considerable distance. Death usually closely follows the wilting of the foliage. In cases where wilting is more gradual,

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FIG. 1. A. Left, wilted sumac tree; right, a smaller healthy tree (×1/10). B. Vascular discoloration (see arrows) in wilted sumac: left, root piece; right, two stem pieces.

dwarfing, chlorosis, and premature autumn coloration may precede wilting. Sporodochia of *Fusarium oxysporum* f. *rhois* sometimes occur in the

lenticels on the bark of wilted trees growing in dense stands on moist sites.

Wilting alone is not always sufficient evidence that the *Fusarium* is present, since a few plants observed with this symptom have been damaged by other causes, such as mildew and boring insects. Wilt-affected sumac has a brown discoloration in the outer sapwood, just beneath the bark (Fig. 1, B). This discoloration is greatest in the roots and lower part of the trunk, and in early stages of the disease may be sparse or lacking farther up the trunk or in the branches. It is often present only on one side in both roots and stem. Vascular discoloration has been found to extend from the roots to the petiole and base of the fruit cluster in some instances. However, there is no evidence to indicate that the fungus is seed-borne. Sections through the discolored vascular tissue show fungus hyphae in the xylem elements.

ETIOLOGY

Fusarium oxysporum f. *rhois* was readily isolated from discolored vascular tissues. Although several morphologic strains of the fungus appeared in laboratory cultures, only one morphologic strain has been isolated from nature, and this one was used throughout the pathogenicity tests.

The pathogenicity of *Fusarium oxysporum* f. *rhois* was tested by a series of experiments. In experiment 1, seed from a wilting staghorn sumac collected in August 1947 was treated with sulfuric acid and planted in the greenhouse in flats containing a mixture of sand and vermiculite. When the seedlings were 1 month old, 13 of them in one flat were inoculated by pouring a few milliliters of a spore suspension of *F. oxysporum* f. *rhois* into a small hole made in the soil near the base of each plant. Another flat containing 20 seedlings from this same seed source served as a control. First symptoms of wilt appeared in the inoculated seedlings after 3 weeks, and all inoculated seedlings were dead within 2 months, while controls remained healthy (Table 1). The *Fusarium* used as inoculum in the soil was

TABLE 1.—*Susceptibility of different species of Rhus to Fusarium oxysporum f. rhois*

Experiment	Age of stock	Tree species	Inoculated		Control	
			Total trees	Trees wilted[a]	Total trees	Trees wilted
			Number	*Number*	*Number*	*Number*
1	1 month	*Rhus typhina*	13	13	20	0
2	1 year	Do	30	22	10	0
3	2 years	Do	3	3	.....	.....
4	4 months	*R. typhina*	18[b]	13	6	0
	4 months	*R. glabra*	18	0	6	0
	4 months	*R. copallina*	18	0	6	0
5	6 months	*R. typhina*	18	10	6	0
	6 months	*R. glabra*	18	0	6	0
	6 months	*R. copallina*	18	0	6	0
6	1 year	*R. toxicodendron*	15	0	5	0

[a] Original fungus reisolated from wilted trees.
[b] All 18 trees showed vascular discoloration at end of experiment.

reisolated from the vascular tissue of all of the wilted seedlings, and microscopic examination showed mycelium present in the vascular elements of the wilted plants.

Seed from healthy *Rhus typhina* in the wilt area of Virginia were used in experiment 2. Ten seedlings were planted in September 1947 in each of four metal flats in a sand and loam mixture, and allowed to grow in the greenhouse. In April 1948, the seedlings in three of the flats were inoculated as in experiment 1. Those in the fourth flat served as controls. The results to date appear in table 1. First wilt symptoms were observed in less than 1 month after inoculation. After 2 months, 7 of the 20 trees in two of the three inoculated flats had wilted and other trees were somewhat dwarfed and chlorotic (Fig. 2).

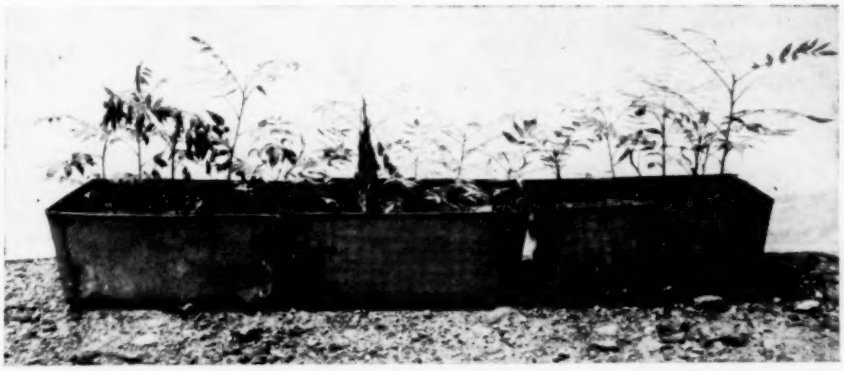

FIG. 2. No inoculum was added to soil in the flat on the right. Two months before the photograph was taken, inoculum was added to soil in the two flats on the left; 7 of the 20 trees in these two flats had wilted at time of photographing.

In August 1947, three 2-year-old plants of *Rhus typhina* growing in North Carolina several hundred miles south of the Virginia wilt area were transplanted to a sand bench in the greenhouse. These trees were allowed to grow undisturbed until April 1948, at which time they were used in experiment 3. All 3 trees were inoculated by pouring a few milliliters of a spore suspension of *Fusarium oxysporum* f. *rhois* over the sand near the base of the trees. All had wilted in 3 months (Table 1).

Experiment 4 was undertaken to compare the susceptibility of *Rhus glabra* L. and *R. copallina* L. with *R. typhina*. On April 15, 1948, six 4-month-old seedlings of each of the three species were transplanted to each of four flats containing a mixture of sand and vermiculite. The seedlings in three of the flats were inoculated with *Fusarium oxysporum* f. *rhois* by dipping the root systems in a spore suspension of the fungus at the time of transplanting. Those in the remaining flat were dipped in sterile water before planting, and served as controls. Two and a half months later, all seedlings were dug up and the root systems examined. Only seedlings of *R. typhina* showed wilt and vascular discoloration (Table 1).

Experiment 5 was a repetition of experiment 4, with additional seed-

lings from the same seedling stock after it had grown 2 months longer. The results agree closely with those of the previous experiment (Table 1).

In experiment 6, the susceptibility of *Rhus toxicodendron* L. to the sumac wilt *Fusarium* was tested. Five 1-year-old seedlings were planted in each of four metal flats in a sand and loam mixture on July 31, 1947. The seedlings in three of the flats were inoculated at the time of planting by dipping the roots in a spore suspension of *Fusarium oxysporum* f. *rhois*, while those planted in the fourth flat were not inoculated. One year later, additional inoculum was added to the soil in the inoculated flats. No wilt has appeared after two seasons (Table 1).

The sumac wilt fungus, *Fusarium oxysporum* f. *rhois*, and the mimosa (*Albizzia*) wilt fungus, *F. oxysporum* f. *perniciosum* (Hepting) Toole,[3] appeared identical in culture, and therefore it was considered necessary to cross-inoculate with these two fungi on the two host species to determine if either fungus could infect both hosts. This was done in the fall of 1947 and repeated in 1948. Sumac plants were wilted only by the sumac *Fusarium*, and Albizzia plants were wilted only by the Albizzia *Fusarium*.

DISCUSSION

Inoculation experiments indicate that for the four species of *Rhus* tested, *Fusarium oxysporum* f. *rhois* is specific and will infect only *Rhus typhina*, while *R. glabra*, *R. copallina*, and *R. toxicodendron* remain unaffected by it. Likewise, this fungus will not infect *Albizzia julibrissin* Durazz., nor will the Albizzia (mimosa) wilt fungus infect staghorn sumac, although the two fungi can not be distinguished from each other in culture. Age of stock apparently had little effect on the development of the disease in these experiments; however, further experimentation is needed to determine the full effect of age of stock as well as soil texture, temperature, moisture, and other factors on disease development.

There appears to be a difference in wilt-susceptibility among different plants of *Rhus typhina*. All of the plants inoculated in experiments 1 and 3 wilted in 3 months, while 73 per cent had wilted after 6 months in experiment 2, 72 per cent in 2½ months in experiment 4, and 55 per cent after 4 months in experiment 5. Because of this apparent variability in resistance, the selection and propagation of wilt-resistant clones of *R. typhina* may be possible. Root cuttings of this species root readily.

The fact that the check plants grown from seed from a wilting sumac remained healthy indicates that this disease may not be seed-borne. The limited geographic distribution of the disease makes it difficult to evaluate its importance at this time. However, it is interesting to note that this is only the second Fusarium vascular wilt of a woody plant species yet reported.

SUMMARY

A vascular wilt of staghorn sumac (*Rhus typhina*) caused by *Fusarium*

oxysporum f. *rhois* has been found in Virginia and Connecticut. It is characterized by wilting of the foliage, soon followed by death of the affected plant. Inoculation experiments indicate that this fungus will wilt *R. typhina,* but not *R. copallina, R. glabra, R. toxicodendron,* and *Albizzia julibrissin,* and that possible strain differences exist in *R. typhina.*

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PHYTOPATHOLOGICAL NOTES

Zonate Leaf Spot of Sorghum in Venezuela.[1]—In September 1947 in plots of grain sorghum (*Sorghum vulgare* Pers.) in Maracay, Aragua, Venezuela, a new kind of leaf spot was found. Dr. C. O. Grassl of the United States Department of Agriculture had sent the seed to Venezuela for experimental purposes, with the advice that it be treated with a dust fungicide before planting; this, however, was not done. The sorghums under test had been collected in Asia and Africa and multiplied in Florida or at Beltsville in the United States. Later this disease became quite common and could be found in other places also. The lesions were subcircular spots with alternating dark and light bands and, in the first hours of the morning, a watersoaked halo. In a moist chamber and within 24 hr., yellow-gray clusters of conidiophores in longitudinal lines appeared in the light zones. These cespitula were minute, pinkish columns, formed by a number of filiform, pluriseptate, arcuate, non-branched, hyaline conidia, which were upright and parallelly juxtaposed on short conidiophores. These characters suggested that the fungus was *Glococercospora sorghi* Bain and Edg., described by these authors[2] as the cause of the zonate leaf spot of sorghum and other grasses in the United States. Dr. Lindsay S. Olive kindly confirmed the diagnosis.

The measurements of conidia were taken in lactophenol, after the leaves had been held for 10 days in a moist chamber at room temperatures (14°–27° C.). The average length of the conidia was 102.5 μ with 139.0 μ maximum and 46.5 μ minimum. The width was from 1.4 μ to 3.0 μ. In other observations conidia of 148.0 μ were seen. Some differences were observed in the length of the conidia, that showed a narrower range than in the type material, and in the unreliability of the slimy matrix about the conidia as a distinguishing feature. The latter character appeared late in the moist chamber and was not seen in the field, possibly because of environmental conditions in Venezuela. In potato-dextrose-peptone agar, slimy conidia were easily obtained. In the field sclerotia were not seen, probably because the leaves decay quickly in these tropical regions and the parasite has not sufficient time to differentiate the resting stage. Sclerotia (80–190 μ), however, formed in naturally infected leaves when these were kept in the moist chamber for a sufficient length of time; and on potato-dextrose-peptone agar, sclerotia appeared within 10–12 days.

Differences in susceptibility to the fungus were noted among the different strains and populations of sorghum grown in the same experimental plot. With two varieties, Milo and Shrock, periodical plantings were

[1] Acknowledgments are made to Dr. Clifford H. Meredith, Head, and Dr. J. H. Standen of the Division de Fitopatología for assistance in the preparation of the English manuscript.

[2] Bain, D. C., and C. W. Edgerton. The zonate leaf spot, a new disease of sorghum. Phytopath. 33: 220–226. 1943.

made throughout the year. The infection was especially severe during September, October, and November, gradually became milder in later plantings, and in the driest months from January through March the disease disappeared completely. It was seen again with the new rainy season in June.

This is the first report of *Gloeocercospora sorghi* in South America. Outside of the United States there is a previous record in Tanganyika, Africa, which seems to be based on sclerotia only.[3, 4]—ANTONIO CICCARONE, Division de Fitopatología, Ministerio de Agricultura y Cría, Maracay, Venezuela.

Sporulation of Gibberella zeae on Rice Straw.[1]—Head scab of wheat, caused by *Gibberella zeae*, is a common disease in humid regions in China and may cause considerable damage.[2] One of the most commonly grown varieties of wheat, No. 2905, which yields well and possesses other good agronomic characters, is very susceptible to scab.

The most practical measure for control of head blight is the growing of resistant varieties, but in testing varieties for resistance to scab, an abundance of spores is essential for good infection. Dickson[3] reported that *G. zeae* produced macroconidia readily on potato-dextrose agar, and Wollenweber[4] stated that conidial or perithecial stages of this fungus were found on various substrates. Apparently the Chinese isolates of *G. zeae* are different from the European and American isolates, because Chinese workers have failed to secure sporulation in various media. In this study conditions conducive to production of conidia were ascertained.

The isolate of *G. zeae* used was collected in Chengtu, Szechwan Province, in April, 1939. Sporulation on eight natural substrates was studied: cabbage, potato, tomato stem, eggplant stem, wheat kernel, cotton stem, and sweet potato cylinder. Fresh material was sterilized directly, but whenever dry plant parts were used they were soaked in tap water for two hours before sterilization. A definite amount of material taken from each of five flasks, incubated at 24° C. for 12 days, was examined for macroconidia. At this temperature the fungus formed white and pinkish mycelium with numerous microconidia on all substrates except wheat kernels. No macroconidia were observed in any of these cultures.

The cultures were kept for a longer time at 24° C. and examined at

[3] Wallace, G. B., and Maud M. Wallace. Tanganyika Territory fungus list: recent records. VI. Mycol. Circ. Dept. Agr. Tanganyika 15. 1945.

[4] Wallace, G. B. Report on a plant disease survey in the Lake and Western provinces and on the Central Railway. March–April 1945, by the plant pathologist. Mycol. Circ. Dept. Agr. Tanganyika 7. 1945.

[1] This work was done at the University of Nanking.

The writer is indebted to Professors C. T. Wei and C. K. Lin for their directions during the progress of the work.

[2] Wu, Y. S. [A report of a survey of wheat diseases.] In Chinese. Nung Pao 3: 1835–1889. 1936.

[3] Dickson, J. G. Production of conidia by *Gibberella saubinetii*. Jour. Agr. Res. [U.S.] 19: 235–237. 1920.

[4] Wollenweber, H. W. Studies on the Fusarium problem. Phytopath. 3: 24–50. 1913.

intervals of five days. On December 26, 1940, 45 days after inoculation, many macroconidia were found on the rather dry sweet potato cylinder. Cultures on other media failed to produce macroconidia within two more months.

In other tests substrates made from corn kernels, corn cob, barley kernels, barley chaff, wheat straw, wheat rachis, and rice straw were inoculated. After incubation at 24°–26° C. for 4 days, macroconidia were present on the rice straw culture. Results were the same in repeated tests.

Cultures on rice straw were incubated at room temperature (approximately 12° C.), 16°, 20°, 24°, 28°, and 32° C. for 20 days. They were examined for sporulation at intervals of two days. Between 16° and 28° C. cultures sporulated rapidly and macroconidia were abundant; at 12° C. sporulation was delayed and few macroconidia were formed; and at 32° C. cultures failed to grow.

In the spring of 1941 cultures grown on rice straw in flasks at approximately 28° C. yielded few macroconidia and it was believed that the medium became too dry because of excessive evaporation. To overcome this, flasks were prepared, containing 25 cc. of 1 per cent agar; after sterilization, in each flask 1 gm. of dry rice straw with 1 cc. of tap water, both previously sterilized, were placed on top of the solidified agar, then two drops of spore suspension containing 10 to 15 macroconidia of *G. zeae* were added as inoculum. The cultures were incubated at 28° C. for 27 days. Three flasks were sampled and macroconidia were counted every third day. Each flask was shaken thoroughly after receiving 20 cc. sterile distilled water, and 5 cc. of the spore suspension were withdrawn for microscopic examination. Macroconidia were counted in five fields under the low-power objective and means were determined for the 15 counts from triplicate samples. Spores were most numerous between the sixth and fifteenth days, but counts declined thereafter.

To secure quantities of macroconidia of Chinese isolates of this fungus, the writer suggests that it be grown for 9 to 18 days on rice straw at 28° C. with sufficient moisture.—S. T. CHEN, Department of Agronomy, College of Agriculture, National University of Shantung, Tsingtao, China.